Characterization of genetic events involving IgH switch regions in gastric low-grade MALT lymphomas and B-CLL

Thesis

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CHARACTERIZATION OF GENETIC EVENTS INVOLVING IGH SWITCH REGIONS IN GASTRIC LOW-GRADE MALT LYMHPOMAS AND B-CLL

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ABSTRACT

This thesis adresses immunoglobulin heavy chain switch regions and variable regions in two hematologic B-cell tumors, mucosa-associated lymphoid tissue (MALT) lymphoma and B-cell chronic lymphocytic leukemia (B-CLL).

A high proportion of IgM+ MALT lymphoma cases were found to bear genetic alterations in the switch regions. Most of these switch region genetic alterations have been characterized through molecular cloning as chromosomal insertions and internal deletions but no new gene has been identified. Variable regions analysis revealed the presence of somatic hypermutations in all cases and intraclonal variation in a proportion of these. Correlation of somatic hypermutations and intraclonal variation with switch region alterations raised the possibility of two distinct subsets of MALT lymphoma with different maturation: one subset with aberrant isotype switch and no intraclonal diversification, and the other one with no aberrant isotype switch but with intraclonal diversification. Replacement versus silent mutation ratio analysis of complementarity-determining regions and frameworks indicated the positive selective pressure of an antigen in most cases. In two cases, protein translated from the third complementarity-determining region suggested the selective pressure of an autoantigen. A correlation of switch region alterations with clinical behaviour revealed that cases with rearrangements have a longer disease-free time after gastrectomy.

In B-CLL switch region analysis demonstrated the presence of switch region alterations in a proportion of cases (34%). These alterations are in most cases internal

deletions of the switch mu region and their role remains to be determined although their localization in the V_HDJ_H rearranged alleles suggests a role in the stabilization of the isotype of the expressed immunoglobulin. Variable regions analysis showed somatic hypermutations in a proportion of cases and no correlation with switch region deletions was detected. No significant correlation between switch region deletions and prognosis was observed, indicating that internal deletions have no consequence for disease outcome. Moreover no significant correlation between the presence of switch deletions and somatic hypermutations was found, indicating that the two processes giving rise to internal deletions and somatic hypermutations, are independent in B-CLL.

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Glossary of abbreviations

MALT	mucosa-associated lymphoid tissue
B-CLL	B-cell chronic lymphocytic leukemia
bp	base pair
dNTPs	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
Ds	double stranded
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte macrophage colony stimulating factor
IFN	interferon
IL	interleukin
Kb	kilobase
MAb	monoclonal antibody
Ig	immunoglobulin
HSC	hematopoietic stem cell
NK	natural killer
BM	bone marrow
Ags	antigens
Abs	antibodies
PBMC	peripheral blood mononuclear cell
MHC	major histocompatibility complex
PC	plasma cell
APC	antigen presenting cell
TCR	T-cell receptor
PTK	protein tyrosine kinase
Th	T helper
DAG	diacylglycerol
IP3	inositoltriphosphate
ECM	extracellular matrix
GC	germinal center
TdT	terminal deoxyribonucleotide transferase
GI	gastrointestinal
V	variable

D	diversity
J	joining
C	constant
S	switch
Nf-kB	nuclear factor kB
RSS	recombination signal sequences
CSR	class switch recombination
AID	activation-induced cytidine deaminase
NHEJ	non-homologous end joining repair
SHM	somatic hypermutation
LG	low grade
HG	high grade
HCL	hairy cell leukemia
PBS	phosphate buffered saline
BSA	bovine serum albumin
RNaseA	ribonulease A
SDS	sodium dodecyl sulphate
FL	follicular lymphoma
Eμ	enhancer
R	replacement
S	silent
FR	framework
CDR	complementarity-determining region
PCR	polymerase chain reaction

Chapter 1

INTRODUCTION

1.1 Blood cell biology

Blood cells grow, divide and differentiate in the bone marrow (BM) through a process called **hematopoiesis**. Three general classes of cells are produced: **red blood cells**, responsible for oxygen transport, **platelets**, responsible for the control of bleeding and **white blood** cells mostly involved in host defence (Figure 1). All three classes are derived from a pool of pluripotent hematopoietic stem cells (HSC) which reside in the BM and are self-renewing cells; when they proliferate some of the daughter cells remain as HSC so that the pool never becomes depleted. In most cases when these cells differentiate their capacity for replication and self-renewal declines and they are called "terminally differentiated". The mature cells of the myeloid lineage include **neutrophils, monocytes, mastcells, eosinophils, basophils** and **megakaryocytes** which produce **platelets**. Mature cells of the lymphoid lineage include **B and T lymphocytes** and **natural killer** (NK) cells. The bone marrow hematopoiesis is controlled at several levels in order to maintain an available pool of HSC, regulate the commitment, proliferation and differentiation of cells and modulate the activity of each pathway in response to physiologic demands. Much of this regulation is achieved through physical interactions of the hematopietic cells with other cells and with soluble factors in the surrounding tissue. By activating specific signal transduction pathways inside the developing cells the external stimuli modulate the activity of transcription factors, cell cycle regulatory factors, and other

intracellular proteins that determine whether a cell will proliferate, differentiate or die (Ogawa, 1993; Uchida et al., 1993; Kincade et al., 1989).

Human HSCs are $CD34^{+}CD38^{-}$, are small with round nuclei and scant cytoplasm and comprise no more than 0.01% of all cells in the BM. Most are mitotically inactive and when cultured in vitro can be induced to proliferate by treating with cytokines like interleukin 3, interleukin 5, granulocyte colony stimulating factor, macrophage colony stimulating factor, granulocyte-macrophage colony stimulating factor etc. When progenitor cells are deprived of an essential cytokine they cease growing and often die though apoptosis.

1.2 Lymphocytes and lymphoid tissues

The typical lymphocyte is a small, round cell, 5-12μm diameter, with a spherical nucleus, densely compacted chromatin and scant cytoplasm. The cytoplasm contains scattered mitochondria and free ribosomes but lacks any distinctive organelles. Several types of lymphocytes can be distinguished by functional properties and proteins expression. The most fundamental distinction is the division into T, B and NK cells. The proportions of T and B cells vary among tissues; in peripheral blood T cells account for 75% and B cells for 10% of all lymphocytes. The remaining 15% are NK cells.

T and B cells derive from the BM HSC following different pathways of maturation. B cells mature uniquely in the BM while T cells undergo part of the process in the thymus. Thymus and BM are the **primary lymphoid organs. Secondary lymphoid organs** serve for the encounter between lymphocytes and foreign antigens (Ags). If

lymphocytes do not encounter any foreign Ag they die within few days; otherwise activation occurs and permits life for several years (Butcher and Picker, 1996).

1.2.1 B cells

B cells defining feature is their ability to synthesize immunoglobulins (Igs) which are called antibodies (Abs) and are able to bind the antigens (Ags). In resting B cells Igs are expressed only on the surface, where they serve as receptors for specific antigens. When an activated B cell divides, some of its progeny become memory B cells and the remainder differentiate into plasma cells (PC) which secrete large amounts of Igs into the surrounding media. PC have oval shape and abundant cytoplasm. The protein-secretory organelles are well represented, including a large Golgi apparatus and abundant endoplasmic reticulum. Lifespan is short, usually days or a few weeks. B cells main function is production of Igs which constitute the humoral immunity but they function also as antigen presenting cells (APCs) and secrete certain lymphokines (Rolink and Melchers, 1991; Sprent and Tough, 1994).

1.2.2 T cells

T cells do not express Igs but instead detect the presence of foreign substances by way of surface proteins called T-cell receptors (TCRs). TCRs are closely related to Igs in evolution but they are never secreted and require direct contact with a target to have activity. Together with macrophages T cells are involved in cell-mediated immunity. T cells will recognize proteins only if cleaved into small peptides displayed on APCs. Specialized APCs are mainly macrophages and B lymphocytes

and presentation occurs through specific proteins forming the major histocompatibility complex (MHC) on the cell surface. Binding of foreign substances to MHC is non-covalent and TCRs must encounter the complex to produce immunologic effects. TCRs are made up of two chains (usually α and β) and are always expressed in association with other transmembrane peptides forming the CD3 complex. This complex transmits signals onto the cytoplasm and is essential for TCR surface expression. CD3 is expressed late in T cell ontogeny. CD2 appears instead at an earlier stage and is useful as a marker for T cell lineage. Different T cell populations exist each with their specific role. $CD8^+$ T cells constitute about 25% of all PB T cells and have cytotoxic activity against cells carrying foreign molecules on their surfaces (i.e. cells infected by viruses). $CD4^+$ T cells are about 70% of all T cells and function as T helper (Th) cells which promote proliferation, maturation and immunologic function of other cell types. A small proportion of T cells (4%) residing outside the thymus are double negative ($CD4^-$ and $CD8^-$) and express a different form of TCR with $\gamma\delta$ chains. Double positive cells ($CD4^+$ and $CD8^+$) constitute only 1% of the T cell population. When activated, T cells can undergo several divisions to produce daughter cells which become effector cells with helper and cytotoxic activity. Effector cells have more cytoplasm and looser chromatin in addition to specific surface proteins such as CD25, CD28, CD29 CD40L and MHCII. When the stimulus is withdrawn, the effector activity declines over a period of several days and the T cells either die or revert to the resting state (Sprent and Tough, 1994).

1.2.3 Lymphocyte activation

Lymphocyte activation occurs through the binding of specific ligands to receptors on the lymphocyte surface and determines proliferation and expression of immunologic functions. Ligands for T and B cells are different but the activation process is very similar. The earliest event is an increase in protein tyrosine kinases (PTKs) within a few seconds of activation. One immediate effect of the PTK cascade is the activation of the phospholipase C-γ1 which then hydrolyzes specific phospholipids in the cellular membranes producing diacylglycerol (DAG) and inositoltriphosphate (IP3). These are second messangers; DAG activates protein kinase 3 and IP3 determines a rapid increase in Ca++ ions. Within the first hour after stimulation the DNA, RNA and protein synthesis rise significantly. In parallel the morphology of the cell changes in a process called blast transformation (Weiss and Littman, 1994; Gold and DeFranco, 1994).

1.2.4 Thymus and bone marrow

The **thymus** is involved in T lymphocyte production and maturation and it is composed of two lobes, each comprising multiple lobules. Lymphocytes are packed densely toward the periphery which assumes the appearance of an outer cortex. Apart from epithelial cells, a few macrophages and other supporting elements, all cells in the thymus are T lymphocytes, also called thymocytes. Of these 10% are CD4- CD8- , have a high mitotic rate and are thought to be T cell precursors. 15% are CD4+ or CD8+ and are preparing to leave the organ. The remaining population is CD4+ CD8+ and represents an intermediate stage of T cell development. Most of them die because of a rigorous selection essential to create a functional immune system but

those which survive become single positive and then leave to the secondary lymphoid organs.

The **bone marrow** can be thought of as a solid tissue in which different types of HSCs develop in physically different locations. These microenvironments are visible in histologic sections of BM which reveal microscopic foci, each devoted to the production of a particular cell type. All is set up and mantained by BM stromal cells. BM cells are able to interact with one another or with proteins and other substances that make up the extracellular matrix (ECM). Hematopoiesis depends not only on specific cytokines but also on a group of cell surface macromolecules known as adhesion molecules (integrins, selectins and various forms of CD44).

1.2.5 Lymph nodes, spleen, tonsils, Peyer's patches,

Lymph nodes are bean-shaped organs distributed along the entire lymphatic vasculature, especially in the neck, axillae, inguinal regions and root of the mesentery. They contain aggregates of lymphocytes and macrophages and connective fibers called reticulin and function as a physical filter where immune cells survey for microrganisms and macromolecules that may be present. The node is surrounded by a fibrous capsule. Lymph flows into the node along afferent lymphatic vessels and enters a subcapsular sinus, lined primarly by macrophages. Then it percolates sequentially through two regions called cortex and medulla and finally exits through an efferent lymphatic vessel on the opposite site. The cortex contains the lymphoid follicles composed mainly of memory B cells, a small number of T cells and follicular dendritic cells. Lymphoid follicles can disappear and reappear over time and can enlarge in response to immune challenges. They are of two types:

primary follicles, containing mature resting B cells and secondary follicles or germinal centers, containing mature B cells in the mantle zone and developing B cells in the light zone (Szakal et al., 1989; MacLennan, 1994). Proliferating B cells in the **germinal center** undergo class switch recombination and a process called affinity maturation.

The **spleen** is located below the diaphragm on the left side of the abdomen and filters blood as the lymph nodes filter lymph. Blood enters through the splenic artery and passes through a network of smaller arterioles encased in a cuff of lymphoid tissue consisting of mature T cells. Primary and secondary lymphoid follicles, composed mainly of B cells, protrude from the lymphoid tissue. All this constitutes the white pulp and permits the flow of blood to the red pulp which is formed by reticular cells and macrophage-lined vascular sinusoids. Eventually blood exits by way of the splenic vein. Flowing through the spleen, blood cells recognize infectious agents, in addition to recognizing abnormal or damaged red or white cells.

Tonsils are aggregates of macrophages and lymphoid tissue located beneath the epithelium of the nasopharynx and soft palate. They detect and respond to pathogens in the respiratory and alimentary sections.

Peyer's patches are lymphoid nodules present in the ileal submucosa of the small bowel where they detect substances that diffuse across the intestinal epithelium.

1.2.6 The mucosal immune system

The mucosal immune system is composed of lymphoid tissues associated with mucosal surfaces of the gastrointestinal (GI), respiratory and urogenital tracts. It is characterized by the presence of a mucosa-related IgA, T cells with specific

properties and a homing system that allows lymphocytes to migrate to subepithelial-tissues. The primary function is to provide host defences at mucosal surfaces where it operates in concert with resident bacterial flora. It provides substances such as gastric acid and secretions that create an unfavorable environment and inhibitory subtances such as lysozyme that inhibit specific microorganisms. Another important function is to prevent the entry of foreign antigens into circulation avoiding an inappropriate immune response.

1.2.6.1 Mucosa-Associated Lymphoid Tissue (MALT)

All lymphoid tissue of submucosal regions is called Mucosa-Associated Lymphoid Tissue (MALT) which secretes antibodies as a defence against external pathogens. It appears that MALT has evolved to protect the mucosal tissue which is in contact with antigens in the external environment. MALT of the GI tract comprises four lymphoid compartments: the Peyer's patches, the lamina propria, the intraepithelial T lymphocytes and the mesenteric lymph nodes. Briefly, the Peyer's patches are composed mainly of B cells, are not capsulated and lack afferent lymphatics; their structure consists of a central follicle surrounded by a marginal zone with a dome epithelium. The lamina propria is composed of plasma cells, B and T lymphocytes, macrophages and APCs. Intraepithelial T lymphocytes consist mostly of CD8+ T cells and mesenteric lymph nodes form the interface between MALT and the peripheral immune system. Antigens enter the Peyer's patches from the gut by a direct mechanism involving specialized "M" cells and stimulate MALT B cells that leave the mucosa and enter the circulation. These B cells then come back to the gut and constitute the lamina propria plamacells.

1.2.6.2 IgA

The mucosal system is rich in IgA which has properties allowing it to function efficiently in the mucosal system. The main properties of IgA are: polymerization and interaction with secretory components, resistance to proteolysis, anti-inflammatory and pro-inflammatory properties. IgA exists in two forms: IgA1 mainly circulating and IgA2 abundant in secretions of the GI tract. Circulating IgA1 is generally a monomer, whereas mucosal IgA1 and IgA2 are generally dimers or multimers. The different properties between the two IgAs are confered by 15-20 aa in the constant domains. IgA1 has a long proline-rich hinge region that confers flexibility to the antigen-binding site but also susceptibility to the proteinases secreted by numerous bacteria. In addition, it has more galactose residues which bind a receptor on hepatocytes. IgA2 displays more truncated oligosaccharides which bind certain organisms which might colonize the mucosal surfaces. Finally, IgA2 has two allotypic forms designated A2M2 and A2M1. In humans, most circulating IgA is produced in the BM in the form of IgA1 monomers whereas secretory IgA is produced mainly at mucosal sites either as IgA1 or IgA2 dimers or polymers.

Why do IgAs develop mainly in mucosal tissues? One factor is the presence of activated T cells that influence B cells to undergo IgA-specific isotype switching (McIntyre et al., 1995). These cells are able to produce TGFβ which promotes IgA switching in addition to expression of CD40L and other not yet identified surface molecules necessary for IgA switch differentiation (Mestecky and McGhee, 1987).

1.2.6.3 Other Igs in the mucosa

Igs other than IgA also play a critical role in the mucosal immune system. IgM can be transported across epithelial cells, is measurable and physiologically significant. It is an adequate replacement for IgA in individuals with selective IgA deficiency. IgG is quite low and can not be transported across the epithelium, but is an important antibody in pulmonary secretions. IgE is synthethized in mucosal tissue particularly during parasitic infection or in relation to allergic states.

1.3 Immunoglobulins and Immunoglobulin genes

Immunoglobulins (Igs) are a huge family of related glycoproteins with various functions. The two hallmarks of Igs as antigen-binding proteins are the specificity and the diversity. In addition, however, Igs also possess secondary biologic activities that are critical for host defence, including the ability to function as opsonins, to activate the complement cascade or to cross the placental barrier. Every Ig is made of two different types of polypeptide: the heavy chains and the light chains. These chains are held together by noncovalent forces and by disulfide bridges. The sequences of the chains show great variability in the N-terminal domain which is called the variable region (V region). The other domains are called constant domains (C domains). Five classes of constant heavy chain (γ, α, μ, δ, ε) and two types of light chain (K and λ) are expressed by humans, with different subclasses and molecular weight. Igs of all classes can exist in either membrane-bound or secreted forms. The membrane forms contain at the C terminus 12-14 acidic amino acids followed by a hydrophobic sequence of 26 residues which anchors the heavy chain into the cell membrane. This direct anchoring occurs only in B cells. Secreted Igs

lack the terminal transmembrane segment as a result of alternative RNA splicing. They contain instead terminal sequences called tail pieces that mediate polymerization of four chain units and also serve as contact sites for the J chain.

1.3.1 Biologic activity

The Ag binding activity is localized in the VH and VL domains and the other activities in the CH domains.

IgGs account for about 75% of the total serum Igs in adults and are the most abundant Abs produced during secondary humoral immune response. In particular, IgG1 is the most concentrated followed by IgG2, IgG3 and IgG4. IgGs are the only ones to cross the placenta in humans and are responsible for protection of the newborn during the first months of life. IgG-Ag binding activates the serum complement. Finally, macrophages and other cell types express receptors that bind the Fc regions of IgG molecules (Spiegelberg, 1974).

IgA. IgA is the predominant Ig produced by B cells in Peyer's patches, tonsils and other submucosal lymphoid tissues although it accounts for only 10-15% of serum Ig. On B cells surface IgA exists as monomer while the predominant secreted forms are dimers and trimers. The two subclasses, IgA1 and IgA2, are expressed at a 5:1 ratio in the blood and have similar properties.

IgM. IgM constitutes about 10% of serum Ig and is normally secreted as a pentamer with a mass of 900 kD. It predominates in the primary immune response and tends to dilute subsequently. It is the most common Ig on B cells and it is also the most efficient complement-fixing Ig.

IgD. IgD is a monomeric four chain unit coexpressed with IgM on the membrane of naïve B cells. Both classes are expressed by alternative splicing of a single mRNA and have identical Ag specificity. It is rarely secreted in significant amounts and its physiologic function is unknown. Its presence on naïve B cells has suggested a role in differentiation or tolerance.

IgE. It is very important from the clinical standpoint because it is involved in allergic disorders, even though it is present at very low concentrations (0.004%). Indeed, inflammatory cells, such as mast cells and basophils, carry a high affinity Fc receptor specific for IgE antibodies. Thus, the surfaces of these cells are decorated with IgE that serve as Ag receptors. When IgEs contact an Ag, inflammatory cells release substances that produce many manifestations of allergic disease.

1.3.2 Human Ig loci

Genes encoding Igs are dispersed along genomic DNA and only developing lymphocytes possess the enzymatic machinery to undergo the process of Ig gene rearrangements.

Light chain genes. All genetic information to produce **kappa chains** lies on chromosome 2 (Figure 1.2-A). The constant domain is encoded by one exon (C_K) present in single copy. The variable domain, however, is contained in two segments called V_K and J_K. V_K codes for the first 95aa and Jk for the remaining 13aa. Multiple V_K and J_K segments are present: about 35 V_k and 5 J_k. Through V/J joining the V_k and J_k segments become covalentely joined to one another to form a single exon. Transcription begins in one VK segment, passes through the fused exon and CK exon synthesizing a primary transcript which is then spliced to form mature kappa

chain mRNA. **Lamba chain** genes arise from a similar gene complex on chromosome 22. The only difference is that there can be up to six different copies of the CL exon each with a nearby JL segment (Figure 1.2-B).

Heavy chain genes. All heavy chains derive from a region spanning about 700.000 bp on chromosome 14 (Figure 1.3). Each heavy chain is encoded by several exons. For example, the μ constant region is encoded by five exons known as the Cμ sequence; the same happens for the other isotypes. In contrast to the light chain genes, a third type of segment, called the diversity segment (DH), is used in forming a variable heavy chain. This segment codes for two or three aa and lies between the VH and JH segments on the unrearranged chromosome. A B cell must complete a V(D)J joining in order to express a heavy chain. Use of the DH segment and errors of a few nucleotides inserted by the enzyme terminal deoxyribonucleotide transferase (TdT) at the joining sites greatly increase the VH chain diversity. TdT is an enzyme that inserts a few nucleotides of random sequence, called N regions, at the points of joining between the V, D and J segments (Komori et al., 1993). Only the CH region downstream of the V(D)J rearranged exon can be expressed and therefore the gene always produces μ chains when it is first rearranged. Expression of other isotypes occurs only after a subsequent recombination called isotype switch recombination. This recombination occurs at specific sites called **switch regions** which lie in front of C genes (see 1.4.2. for further details). One exception is the Cδ gene which is transcribed with Cμ. This produces an RNA that can be spliced to yield either μ or δ mRNA enabling the cell to express IgM and IgD at the same time. Such coexpression is a feature of naive B lymphocytes.

1.4 B cell development

Lymphocyte development is among the most intensively studied examples of progressive lineage commitment and serves as a prototype of mammalian cell differentiation. Several genes regulate stem cell commitment to lymphoid differentiation. PU-1, a transcription factor of the Ets family, Ikaros, a DNA-binding protein, EBF, a regulator of mb-1 which encodes for Igα, E2A transcription factors. The B cell develops and matures in the BM while differentiates in the peripheral lymphoid organs (Figure 1.4). B cell development in the BM can be defined by expression of a variety of cell surface markers and by the status of Ig gene rearrangements. $CD43^+$ **pro B cells** undergo a first Ig rearrangement becoming **early pre B cells**. These further differentiate into smaller $CD43^-$ **late pre B cells** in which rearrangement of the light chain loci occurs and results in production of surface-expressed Ig. This signal triggers differentiation into **naive (virgin) B cells** which express IgM and IgD. B cells with self-reactive Igs are deleted by induction of programmed cell death within the BM. The remaining cells migrate out of the BM to replenish the peripheral immune system. In the periphery, unstimulated B cells die within a few days while stimulated B cells get activated and differentiate through T cell help in the germinal centers of the spleen and lymph nodes. Within germinal centers, B cells undergo important changes including isotype switch (1.4.2), affinity maturation (1.4.3), plasma cell differentiation and the generation of memory cells (1.4.4) (Arpin et al., 1995; Li et al., 1993).

Germinal centers (GC) are complex structures that form in the spleen and lymph nodes in response to antigenic challenge. In addition to B cells they contain antigen-presenting cells (APCs) and T helper (Th) cells. There are three histologically

distinct microenvironments within the GC. The mantle zone contains small resting B cells that have not yet been antigenically selected. Activation of these B cells induces proliferation and somatic hypermutation which occur in the dark zone. Surviving clones enter the light zone where proliferation slows and affinity selection predominates. GC development remains poorly understood but a number of factors involved have been recently identified. CD19 is a potent costimulator of Ig-mediated signaling and is expressed throughout B-cell development. Bcl-6 is a transcriptional repressor highly expressed by activated B cells. Bcl-3 appears to enhance nuclear factor kB (Nf-kB) DNA binding. Tumor necrosis factor α and β (TNFα and TNFβ) regulate humoral and cellular immune responses and their gene inactivation results in disruption of GCs. Finally, BSAP is an important regulator of early B cell development but also of late stages, during activation and isotype switching. Isotype switching, which also occurs in GCs, allows the production of IgG, IgA, IgE in response to specific cytokines (MacLennan, 1994).

Activated B cells ultimately choose between death or differentiation into **plasma cells** (PCs) or **long-lived memory cells** which constitute the basis for the response to antigenic rechallenge in a secondary immune response. Memory B cells migrate to the bloodstream and PCs to the BM, becoming mature PC.

1.4.1 The mechanism of V(D)J recombination

V(D)J recombination fuses a large selection of gene segments to form functional heavy and light chains generating, through different combinations, great antibody diversity (Oettinger, 1996; Alt et al., 1987; Lewis, 1994). **Recombination signal**

sequences (RSS) are non-coding motifs which flank V and J segments necessary for V(D)J recombination (Akira et al., 1987; Hesse et al., 1989). They consist of conserved heptamer and nonamer motifs separated by 12 or 23bp spacers. (Figure 1.5A). V(D)J recombination in vivo occurs only between motifs with different length spacers (12 or 23) (Tonegawa, 1983). Two proteins, **RAG1 and RAG2,** are essential for V(D)J recombination. Their genes are extremely close in the genome and have the unusual feature of lacking introns (Schatz et al., 1989; Oettinger et al., 1990). RAG1 and RAG2 are able to carry out in vitro the initial steps of V(D)J recombination. These steps occur upon single strand nicking and nucleophilic attack by the resulting 3' hydroxyl on an adjacent phospodiester bond of the opposite strand which causes a double strand break with a hairpin loop on the coding end. These hairpins are intermediates in the process of V(D)J recombination in vivo. One other important enzyme involved in V(D)J recombination is a DNA dependent **protein kinase (PK)** specific for serine and threonine residues, which has the role of repairing double strand DNA breaks (Kirchgessner et al., 1995; Blunt et al., 1995; Peterson et al., 1995). Mice deficient for RAG1 and RAG2 (Mombaerts et al., 1992; Shinkai et al., 1992) or with mutations in DNA-PK (SCID mice) (Bosma et al., 1988) show defects in different stages of B-cell develoment.

When RSS are joined together the DNA that originally separated V and J segments can be released as a covalently closed circle (which is then degraded) or it can be removed by way of an inversion (Figure 1.5B).

Rearrangements occur first on the IgH locus, between D and JH gene segments, and later between VH and DJH. Only after the formation of functional rearrangement of the IgH locus, the IgL locus rearranges, kappa genes preceding lamba genes. The formed receptor associates with two other transmembrane molecules, Ig alfa and Ig

beta. This dimer is required for cell surface expression and signalling which appears to act through activation of the tyrosine kinase **Syk**. This complex represses RAG1 and RAG2 genes although the intracellular components of these signalling pathways have not yet been described. This ordered rearrangement of the Ig genes appears to derive from controlled sequential accessibility to the recombinase machinery. The precise mechanisms governing the accessibility of antigen receptor loci are not fully understood, although the so called sterile transcripts may open the locus by causing displacement of inhibitory or masking proteins (Willerford et al., 1996; Sleckman et al., 1996).

1.4.2 Isotype switching

Isotype switching or class switch recombination (CSR) occurs in activated B cells and permits the exchange of the constant region of the heavy chain from IgM/IgD to IgG, IgA, or IgE. This rearragement occurs by deletional recombination between switch regions located upstream of each constant gene segment and serves to alter the effector function of the antibody molecule. No consensus or homologous sequence is found around switch regions and CSR can be classified as region-specific recombination.

The order of the constant region genes in the IgH locus, starting from the telomeric end, is μ, δ, γ3, γ1, ψε, α1, ψγ, γ2, γ4, and α2. Isotype switch from μ to γ, α, or ε is a developmentally regulated DNA recombination event that occurs between Sμ and the respective isotype switch region, resulting in the formation of a hybrid switch region. The **switch regions** are 1-3 kb long, consist of tandem pentameric repeats, and are located just upstream of μ, γ3, γ1 α1, γ2, γ4, and α2 constant segments. There is no

conventional switch region upstream of δ, ψε or ψγ. However, isotype recombination to δ can occur by homologous recombination between duplicated 442-bp sequences that are located upstream of Sμ and the first exon, respectively (Bergsagel et al., 1996).

A "**physiological**" IgH switch recombination occurs between two IgH switch regions, resulting in the formation of a hybrid switch region. This may be a productive switch from μ to another isotype, a downstream switch not involving μ, or an inversion event between switch regions (Figure 1.6). In most cases, these recombination events occur on the same chromosome (cis), but occasionally occur in trans (recombination between sister chromatids or homologous chromosomes). An "**aberrant**" IgH switch recombination, which is defined as involving only one IgH switch region, may be a chromosomal translocation, an interstitial deletion, an interstitial insertion, or an inversion into a switch region. A shematic representation of the possible switch recombinations is shown in Figure 1.6. When the Sμ region is joined to a downstream switch region the intervening DNA is loopped out and excised as switch circular DNA (**switch circle**) resulting in the expression of the downstream isotype.

The entire process generating switch circles can be divided into 3 steps: choice of a downstream switch region, recognition and cleavage of the target DNA by the recombinase and repair and ligation resulting in switch circles.

1.4.2.1 Selection of the target

Stimulation by a **specific cytokine** determines the target switch (S) region and thus, the immunoglobulin class generated by CSR. In mature B cells, the Sμ region is

constitutively transcribed from the intronic promoter (I) located 5' to the Sμ region, whereas transcription of downstream S regions is induced only after stimulation with specific cytokines (i.e interleukin 4). Each cytokine activates specific intronic promoters that are located 5' to S regions, giving rise to the so-called **germline transcripts** that contain the intronic promoter and the constant exons. Germline transcripts are required for effective CSR and recently it was demonstrated that there is a direct correlation between the amount of germline transcripts and class switch efficiency (Lee et al., 2001).

Splicing removes the S region sequence from primary transcripts. A close association between isotype specificity of germline transcription and the recombination target of S regions by stimulation with a given cytokine has led to the **accessibility model**, whereby germline transcription opens the chromatin structure of a specific S region and renders it accessible to a putative recombinase (Yancopoulos et al., 1986). Several gene-targeting experiments abolishing germline transcription (Gu et al., 1993; Jung et al., 1993; Zhang et al., 1993; Seidl et al., 1998) confirmed that this process is required during CSR. Transcription-dependent locus opening had been also shown to be required for V(D)J recombination (Blackwell et al., 1986).

1.4.2.2 Recognition of target DNA

To understand the cis elements that are required for CSR, various in vitro assay systems were developed using artificial DNA constructs and B cells or B cell lines (Ott et al., 1987; Leung and Maizels, 1992; Lepse et al., 1994; Daniels and Lieber, 1995; Christine et al., 1999; Stavnezer et al., 1999; Kinoshita et al., 1998). One of the most effective was a system using an efficiently switching mouse lymphoma cell line

(CH12F3-2), in which 80% of the cells switch from expressing IgM to (almost exclusively) IgA in one week after stimulation with CD40L and IL-4. Constructs containing Sμ and Sα regions in front of surface expression markers were introduced into CH12F3-2 cells. CSR requires cytokine stimulation, indicating that the stimulation induces activation of new genes involved in CSR such as activation-induced cytidine deaminase (**AID**) (see 1.4.4 for details on this enzyme) (Kinoshita et al., 1998; Muramatsu et al., 2000). Studies using several other types of artificial construct in combinations of B cell lines and spleen cells (Leung and Maizels, 1992; Lepse et al., 1994; Daniels and Lieber, 1995; Ott and Marcu, 1989) have clearly shown that the S region is essential for CSR. Furthermore, knockout mice with disruption of the core S region showed decreased levels of class switching to all isotypes (Luby et al., 2001). Residual switching observed in these mice might be due to the presence of scattered S repeat motifs outside the core S region.

The **class-switch recombinase** might recognize the secondary structure of S regions rather than the primary sequence. Evidence for this idea includes the fact that the S regions of different isotypes and species (chicken and frog), as well as an inverted S region, are functional in CH12F3-2 cells, which switch almost exclusively to IgA. Scrutiny of recombination junctions in mammalian and amphibian CSR — in both endogenous loci (Mussmann et al., 1997) and artificial substrates (Tashiro et al., 2001) — revealed that their distribution is biased to the proximity of the stem–loop structure in single-stranded S sequences, which is predicted by a computer program based on thermodynamic parameters (SantaLucia, 1998). Because S sequences, irrespective of the isotypes and species, commonly contain many short stretches of inverted repeats, the stem–loop structure may be formed when they are single stranded.

1.4.2.3 Cleavage and repair.

Using artificial substrates that direct CSR it was demonstrated that deletions and duplications of variable lengths occur at junction sites (X.C. Chen, K.K. and T.H., unpublished data). The introduction of duplications during CSR indicates that DNA breaks in S regions are due mainly to staggered nicks (double strand break that produces protruding ends) on two strands of DNA. Because cleaved ends are joined by the **non-homologous end joining repair (NHEJ) systems** during CSR, single-stranded tails of staggered ends might be blunt-ended by **exonucleases** and might involve **error-prone DNA polymerases**. During these processes, frequent mutations are introduced in the region surrounding the recombination breakpoints.

In the course of recent studies on Hairy Cell Leukemia it has been proposed that CSR might occur not only with deletions at the DNA level but also through RNA splicing. Indeed HCL cells express simultaneously transcripts of multiple isotypes. This could not be possible considering only the "switch deletional mechanism" because in this model the isotypes located between the μ and the target isotype would be deleted. An RNA splicing event, preceding CSR, would allow the expression of multiple isotypes at the RNA level (Forconi et al., 2001).

Like V(D)J recombination isotype switching is regulated at multiple levels. As discussed above, the enzymatic machinery operates through generation of double-strand breaks and rejoining of the looped-out DNA. Accessibility of switch regions requires germline transcription of the IgH constant genes which is regulated by multiple factors including surface Ig crosslinking, CD40 receptor engagement and soluble cytokines such as IL4, IFN γ, and TGF β.

Four enhancers, located 3' to the IgH locus, strongly affect the efficiency of CSR. It has been demonstrated that Ca3/hs3a and hs1,2 are dispensable for switch recombination and that hs3b and hs4 impair CSR if deleted (Pinaud et al., 2001).

CSR capacity has been tested for various CD27 memory B cells. IgM+IgD- and switched-memory cells have low switch capacity compared with IgM+D+ cells which undergo readily sequential switch recombination when properly stimulated. IgM only cells might undergo abortive switch and switched cells might bear alterations which impair switching capacity (Werner-Favre et al., 2001).

Several important proteins involved in CSR have been characterized recently and the most intriguing aspect is the finding that many of them also have a fundamental role in the process of somatic hypermutation (SHM). The most important are

1) CD40-CD40L intraction with Nf-kB activation
2) NEMO (Nf-kB essential modulator)
3) Activation-induced cytidine deaminase (1.4.3.3)
4) Mismatch-repair proteins

1.4.3 Somatic hypermutation

Somatic hypermutation (SHM), occurring within the follicular GC dark zone, increases the mutation rate of the heavy and light variable regions. This process increases diversity within the antigen-binding pocket of the Ig receptor. Affinity maturation derives from the combination of SHM and stringent antigen-driven selection and results in antibodies with much greater affinity and specifity for target antigens in a secondary immune response. Although it is unknown which strand is targeted for mutation, the mutational process has a strand bias, favouring purine

substitution on the coding strand. The mechanism is not fully understood but it appears quite specific as adjacent regions are not affected. DNA repair proteins have been identified as components of the RNA polymerase II transcription initiation complex. SHM introduces point mutations specifically in immunoglobulin V genes of activated B cells at an extraordinarily high frequency (10^{-3} bases per generation) (Storb et al., 1998; Jacobs and Bross, 2001). This frequency is roughly one million times higher than the level of mutation during normal DNA replication (10^{-9} bases per generation). Not all switched Igs undergo SHM, and conversely, some IgM have SHMs, indicating that neither CSR nor SHM is a prerequisite of the other. SHM occurs even in the absence of CD40-CD40L interaction in patients with hyper IgM syndrome (HIGM). For this reason it is hypothesized that an alternative diversification pathway must exist (Weller et al., 2001).

1.4.3.1 Selection and recognition of the target.

Transcription seems to be essential for SHM. Mutations are commonly found within a 1.5–2-kb region that lies 3' to the promoter region of the rearranged V gene, regardless of families and endogenous or transgenic loci, and mutations upstream of the transcription start site are extremely rare. Moreover, the intronic enhancer and promoter have been shown to be required for SHM (Betz et al., 1994). Because the mutation rate is positively correlated with the transcription efficiency (Fukita et al., 1998; Bachl et al., 2001), it should be carefully examined to find out whether the enhancer sequence is essential for functions other than transcriptional activation, as proposed (Papavasiliou and Schatz, 2000). As in the case of CSR, SHM does not seem to have a clear primary-sequence specificity.

Storb and colleagues (Storb et al., 1998) proposed that transcription is required to recruit a putative **mutator** (possibly a 'nickase'). According to their model, the mutator can bind to RNA polymerase II during initiation of transcription, and be transferred to DNA when the RNA polymerase pauses because of a secondary structure (stem–loop) of the nascent transcript. Kolchanov and colleagues (Kolchanov et al., 1987) speculated that the mutator might recognize non-complementary base pairs in the stem–loop structure of the V-region genes because the abundance of complementary palindromes in V-region genes is about four times higher than that in other genes including immunoglobulin C-region genes.

1.4.3.2 Cleavage

Most models describing SHM propose a break of DNA strand(s) but direct evidence to support DNA cleavage during this process has only recently been obtained (Papavasiliou and Schatz, 2000; Goossens et al., 1998; Wilson et al., 1998; Sale and Neuberger, 1998; Bross et al., 2000; Kong and Maizels, 2001). Indeed deletions, duplications or insertions were found in V genes of germinal-centre B cells. Moreover, the idea that DNA breaks occur during SHM is supported by the observation (Sale and Neuberger, 1998) that short, non-templated additional bases are inserted into a constitutively mutating V gene in a human B cell line (Ramos) overexpressing TdT. Two other groups detected double-stranded lesions in V genes of B cells undergoing SHM (Papavasiliou and Schatz, 2000; Bross et al., 2000).

1.4.3.3 Repair

The final step of SHM — repair of the cleaved ends — should be the key step for introducing point mutations. Many groups have explored the possible involvement of repair enzymes using gene targeting, but the results are confusing. Whereas the deficiency of some proteins showed no effect, deficiency of **mismatch repair proteins** including Pms2 (Cascalho et al., 1998; Winter et al., 1998), Msh2 (Bertocci et al., 1998; Frey et al., 1998; Rada et al., 1998; Phung et al., 1998) and Mlh1 (Kim et al., 1999) caused lower mutation frequencies and/or altered mutation spectra. An interpretation of all available results has led to two opposing views: either that mismatch repair modifies or fixes incipient mutations; or that mismatch repair has no role in SHM. However, it is worth noting that Msh2 deficiency affects the efficiency and local sequence specificity of both CSR and SHM (Rada et al., 1998; Wiesendanger et al., 2000; Schrader et al., 1999; Ehrenstein and Neuberger, 1999). Recently, homologous recombination with the sister chromatid was proposed to be involved in the repair of double-stranded DNA breaks and in the generation of mutations (Papavasiliou and Schatz, 2000). One clear finding, however, is that the NHEJ system is dispensable for SHM (Bemark et al., 2000).

Several DNA polymerases have been recently assigned a role in SHM. Polymease iota and mu, polymerase zeta and polymerase eta (Dominguez et al., 2000; Zan et al., 2001; Rogozin et al., 2001).

1.4.4 Activation-induced cytidine deaminase (AID)

During experiment involving complementary DNA subtraction between switch-induced and non-induced CH12F3-2 cells, a new protein called activation-induced

cytidine deaminase (AID) was identified, which is expressed specifically in B cells that have been activated in vivo or in vitro. In situ hybridization showed that AID expression is restricted to germinal centers, where CSR and SHM take place.

Overexpression of AID in CH12F3-2 B cells enhanced CSR, irrespective of cytokine stimulation. Mice deficient for AID cannot produce IgG, IgA or IgE antibodies, whereas IgM is normally (or even more abundantly) produced under immunized or non-immunized conditions (Muramatsu et al., 2000). Immunoglobulin classes other than IgM and IgD were not detected, even after LPS and cytokine stimulation of spleen cells in vitro. Sequence analysis of the VH186.2 V gene in AID-/-mice after immunization with 4-hydroxy-3-nitrophenylacetyl (NP) conjugated with chicken-globulin (CGG) showed that the mutation frequency was no more than the error rate of the Taq polymerase used for the experiment. A mutation in the CD40L gene has been shown to cause a condition called X-linked hyper-IgM syndrome type 1 (HIGM1), which manifests as severe immunodeficiency owing to a defect in CSR (Allen et al., 1993; Aruffo et al., 1993; DiSanto et al., 1993; Korthauer et al., 1993). There is also another type of HIGM (HIGM2), which shows similar clinical symptoms but is inherited in an autosomal-recessive manner (Revy et al., 1998). Both the human AID gene and the HIGM2 locus have been mapped to chromosome 12p13, and sequencing of the AID coding region from 18 patients with HIGM2 identified various mutant alleles, all of which would give rise to mutant AID proteins with amino-acid replacements or truncations (Revy et al., 2000). Moreover, SHM was not observed in B cells from HIGM2 patients. These findings in human and mouse convincingly demonstrate the requirement of AID for both CSR and SHM.

In vitro stimulation of B cells from AID-deficient mice with LPS and cytokines induced normal germline transcription of downstream S regions. This implies that

AID is involved in neither signal transduction from the cell-surface receptor to the nucleus, nor in the establishment of the accessible state of the S region. V(D)J recombination is normal in humans and mice with the AID mutation, indicating that AID is not involved in the repair process that is shared between V(D)J recombination and CSR. In other words, AID is probably involved in the **cleavage step** during CSR. The predicted amino-acid sequence (198 residues) of AID has homology to that of APOBEC-1, which is a catalytic subunit of the RNA-editing complex for the apolipoprotein B messenger RNA precursor (Chester et al., 2000). APOBEC-1 converts the specific cytosine at position 6666 of apolipoprotein B mRNA to uracil by its intrinsic cytidine deaminase activity, giving rise to mRNA that encodes the CHYLOMICRON component. Although APOBEC-1 cannot bind a specific sequence in the target mRNA, this specificity is conferred by a protein with which it associates, APOBEC-1 complementation factor (ACF) (Mehta et al., 2000). Like APOBEC-1, AID synthesized in vitro has cytidine deaminase activity. Moreover, both the APOBEC-1 and AID genes are closely mapped to human chromosome 12p13. These structural, genetic and functional relationships of AID and APOBEC-1 indicate that AID might be an **RNA-editing enzyme**. AID, in a complex with an ACF-like protein, might edit a certain mRNA, giving rise to a new mRNA that encodes the class-switch recombinase.

1.4.5 Differentiation into memory cells or plasma cells

After isotype switching and SHM, selected germinal center B cells are subject to differentiative signals that drive cells into one of two fates: to become plasma cells or memory cells (Fig. 1.5) (Klinman, 1996; Liu et al., 1997). Differentiation into

plasma cells results in a switch from generation of predominantly membrane-bound Ig to high level production of secreted Ig. Plasma cells produce the vast majority of circulating antibodies. In contrast, memory cells represent long-lived high-affinity resting cells. On rexposure to target antigen these primed cells are able to rapidly amplify and differentiate generating large numbers of Ig-secreting plasma cells. Unlike plasma cell differentiation in a primary immune response, plasma cell differentiation from memory derived precursors does not involve germinal center formation.

Some of the factors regulating these developmental pathways have been identified. The CD40 receptor, among its several other roles in B cell development, has been implicated as a key regulator of the differentiation branch point between memory and plasma cells (Arpin et al., 1995) (Lane et al., 1995; Callard et al., 1995). In addition a number of cytokines have been implicated in regulating differentiation pathways of activated B cells.

1.4.6 Regulation of lymphocyte survival during B-cell development

The lymphoid immune system exists in a state of dynamic homeostasis, with rapid clonal expansion and massive cell turnover in almost all phases of development. Turnover results from induction of apoptosis in the vast majority of lymphocytes and lymphoid progenitors. The apoptotic pathway attenuates immune responses and eliminates autoreactive cells. Deregulation of apoptosis has been associated with immunodeficient states, lymphoma, and autoimmune disease (Rudin and Thompson, 1997).

Bcl-2 was the first identified member of an expanding family of apoptotic regulators (Tsujimoto and Croce, 1986). The t(14;18) translocation found in 85% of follicular B cell lymphomas juxtaposes the Bcl-2 gene with the IgH enhancer, resulting in constitutive overexpression of Bcl-2 and expansion of the mature-appearing B cells characteristic of this disease. Bcl-2 family members may also play important roles in normal lymphocyte maturation. Expression of Bcl-2 and the related apoptotic inhibitor Bcl-xL are differentially regulated during B cell development (Merino et al., 1994) (Grillot et al., 1996). Bcl-xL is expressed in developing pre-B cells, and may be essential for survival through this period of clonal selection. Bcl-2 is expressed in pro-B cells, downregulated during the pre-B cell stage, and re-expressed in mature B cells. Neither Bcl-2 nor Bcl-xL appears to be expressed during the immature B cell stage during which deletion of autoreactive naive B cells occurs. Bcl-2 expression s appears to be critical in mature B cell, as homozygous disruption of the Bcl-2 gene results in relatively normal lymphoid development but progressive loss of the mature B lymphoid immune system (Nakayama et al., 1993) (Veis et al., 1993). Finally, Bcl-xL, but not Bcl-2, is strongly upregulated during B cell activation, and may be essential for the survival and expansion of the responding population.

Progression through the sequential stages of pre-B cell development requires a series of positive survival signals dependent on the production of appropriate membrane-bound receptors. lnability to generate a functionally rearranged IgH gene results in developmental arrest and apoptosis in the majority (perhaps 90%) of B cell precursors. Similarly, failure to generate a functional IgL able to pair with the IgH as surface Ig results in apoptosìs and further reduction in B cell precursors. Newly generated B cells producing an autoreactive IgM are eliminated by apoptosis before

leaving the bone marrow. Only a small fraction of the B cell precursors generated survive to constitute the peripheral immune system.

In the absence of positive stimulation, peripheral lymphocytes are also highly susceptible to apoptotic induction. Unless rescued by exogenously added cytokines, peripheral lymphocytes undergo apoptosis within hours of removal from the body. Unstimulated lymphocytes in vivo survive on average only a few days in the periphery, dying again by apoptosis.

A complex interplay of cell death signals directs germinal center selection (Liu et al., 1997). Lymphocyte activation is associated with dramatic increases in both proliferation and apoptotic sensitivity. The priming of germinal center B cells for apoptosis reflects the upregulated expression of several factors involved in apoptotic induction, including c-Myc, p53 and Fas (Martinez-Valdez et al., 1996). High level expression of c-Myc in particular is associated with both marked proliferative induction and hightened apoptotic sensitivity.

Understanding the pathways between cell surface receptors and the intracellular apoptotic regulators has been a major focus of recent research on lymphocyte activation. A number of cell surface receptors related to the TNF receptors have been found to play critical roles in regulating lymphocyte survival. Of particular importance in B cell survival are the **CD40** and **Fas receptors** (Rathmell et al., 1996; Goodnow, 1996). CD40 interacts with the CD40 ligand expressed on the surface of T cells and conducts a critical costimulatory signal in T cell-dependent B cell activation. Ligand binding to the Fas receptor, in contrast, generates a potent apoptotic signal in lymphocytes. Survival of germinal center B cells depends on at least two signals, generated by productive engagement of the receptor and the CD40 receptor. After SHM, generation of high affinity Ig receptors is required for the first

signal. Antigen processing and presentation by high affinity B cells to germinal center T cells may in turn trigger presentation of the second signal, the CD40 ligand on the surface of activated T cells. B cell stimulation through both the surface Ig and CD40 receptors results in activation, proliferation, and a transient resistance to Fas mediated death. In contrast, CD40 engagement in the absence of antigen recognition by the Ig receptor does not results in activation or proliferation but heightens susceptibility to Fas mediated apoptosis. The requirement for cooperative interaction between antigen-specific B and T cells may help to ensure selectivity and specificity in the immune response.

Ultimately, the vast majority of the selected high affinity B cell clones amplified by an immune response undergo massive apoptosis in the termination of that response. Factors involved in the loss of apoptotic resistance in this process may include elimination of the antigenic stimulus, loss of costimulatory signals, and the delayed expression of additional factors (such as CTLA-4 on activated T cells) that interrupt the costimulatory feedback loop between activated B and T cells. Only a minute fraction of the activated population persists as memory cells.

1.5 Origin and history of B cell tumors as revealed by analysis of immunoglobulin genes

Since the Ig locus plays a fundamental role in the development of B cell tumors Ig-gene status has become an indicator of the tumors' cell of origin. Normal B-cell differentiation is characterized by a series of regulated DNA rearrangements and neoplastic events can occur at many points. Briefly, peripheral B-cell maturation can be divided into 3 stages. In the first stage (pre-GC stage) the naive B cell has not yet

encountered the antigen. In the second stage (GC stage) SHM and isotype switching can occur, following entry into a GC of the peripheral lymphoid tissue (Berek, 1992; Kelsoe, 1995). In the GC, B cells encounter T cells and antigens presented on dendritic cells and produce high affinity antibodies. Unselected cells die of apoptosis. In the third stage (post-GC stage), memory B cells may recirculate or home to the antigen draining sites. Alternatively B cells may differentiate to plasma cells (Klein et al., 1997; Dunn-Walters et al., 1995) (Figure 1.7).

The natural DNA rearrangement occurring during V(D)J recombination or CSR renders chromosome 14 vulnerable to translocations and other rearrangement events (Rabbitts, 1994). Similarly chromosome 2 and 22 may be involved. A further influence on tumor development derives from SHM which targets V(D)J sequences and surrounding translocated genes (Yelamos et al., 1995; Cook and Tomlinson, 1995).

Each tumor carries its variable genes (V genes) as clonal markers and these, identified and sequenced, allow the assignment to histopathological or clinical tumor categories. Identification of clonal V genes in tumors has implications for diagnosis, monitoring and treatment (Matsuda et al., 1993). Importantly, V gene sequences will indicate whether the cell of origin has undergone affinity maturation under the influence of an antigen. The accumulation of somatic mutations in the V_H genes indicates that the cell has been exposed to the hypermutation mechanism and therefore must have entered or passed through the GC (Du et al., 1996; Berek et al., 1991; Hummel et al., 1994; Rajewsky, 1996; Bahler et al., 1991; Levy et al., 1987). The detection of intraclonal diversification allows discrimination between GC B cells, which show non-common mutations, and post GC B cells which do not (Kuppers et al., 1993).

When somatic mutations are detected it is important to define if they are determined by the presence of an antigen. This can be done by studying the distribution of replacement amino acids in the IgH V genes and can be informative since the antigen could drive the proliferation of the tumor (Bahler and Levy, 1992). Preferential V gene usage can reveal a possible role for superantigens which can bind to unmutated framework regions (Li et al., 1996; Silverman, 1992).

As the study of V genes gives important information on the maturation of the tumor's cell of origin, even the study of IgH switch regions can give similar information. It is possible to distinguish cells which have not entered the GC and cells which have entered or passed the GC. The first are "naive" cells and the second are mature or "experienced" cells. Not all experienced cells have undergone CSR (i.e. cells with IgM isotype) but the study of IgH switch regions can still give anyway important information. Frequently, B cells are not able to undergo a complete CSR with isotype exchange (from M to G,A,E) but carry genomic alterations in the switch regions which will indicate an attempt of the cell to undergo CSR.

1.6 Mucosa-Associated Lymphoid Tissue (MALT) lymphomas

Mucosa-Associated Lymphoid Tissue (MALT) lymphomas originate from native or acquired MALT. Native MALT is physiologically present whereas acquired MALT develops in sites of inflammation in response to infections such as *Helicobacter pylori* gastritis, or autoimmune processes such as myoepithelial sialadenitis associated with Sjogren's syndrome or Hashimoto's thyroiditis. In this context a pathological clone can replace the normal lymphoid population and give rise to MALT lymphoma. MALT lymphoma shows the features of primary extranodal

lymphomas that arise outside the lymph nodes and constitute between 25% and 40% of malignant non-Hodgkin's lymphoma. MALT lymphomas can arise from 1) Extranodal lymphoid organs such as the spleen; 2) Non-lymphoid organs containing a native lymphod tissue such as the gastrointestinal tract; 3) Organs normally devoid of lymphoid tissue such as the brain. Clinicopathologic features of MALT lymphomas show that these lymphomas are more closely related to the structure and function of MALT than to peripheral lymph nodes.

1.6.1 MALT lymphoma main features

In 1980, Isaacson and Wright defined a morphologically and clinically homogeneous group of lymphomas (Isaacson and Wright, 1984). They tend to remain localized for long periods and seldom involve the bone marrow at presentation. These lymphomas are often preceded by infections that induce acquired MALT and most commonly occur in the stomach but also in the intestine, salivary gland, respiratory tract, thyroid, ocular adnexa, thymus, liver, genitourinary tract, breast and skin.

1.6.2 Low-grade MALT lymphoma histology

Histologic features of low-grade MALT lymphoma closely simulate those of the Peyer's patches. Lymphoma infiltrates around reactive follicles and spreads into the surrounding mucosa. Tumor cells resemble follicle center centrocytes, small lymphocytes or monocytoid B cells and form lymphoepithelial lesions by invasion of individual glands. Often it is possible to find transformed blasts, plasmacell differentiation and follicular colonization, all histologic features suggesting that the

lymphoma cell participates in an immune response. Involvement of gastric lymph nodes occurs in about 30% of cases (Montalban et al., 1995). In these cases the lymphomatous infiltrate extends to form confluent sheets with eventual replacement of the node

1.6.3 High-grade MALT lymphoma histology

In addition to transformed blasts, it is possible to find in low-grade MALT lymphoma foci of high-grade transformation characterized by confluent clusters of transformed blasts (De Jong et al., 1997). Isolated foci of typical low-grade MALT lymphoma can be found in high-grade cases, indicating the possibility for low-grade to transform into high-grade. In the absence of low-grade features, it is very difficult to differentiate high-grade MALT lymphomas from other high-grade B cell tumors. Only the characteristic lymphoepithelial lesions may be found. High-grade cells may resemble centroblasts or plasmablasts and sometimes may present numerous multinucleated forms. The close association with *H pylori* infection and the absence of Bcl2 rearrangements may contribute to the classification of high-grade MALT lymphoma.

1.6.4 Immunophenotype

B cells of MALT lymphoma express surface immunoglobulin, usually of the IgM isotype, which shows light-chain restriction (Griffin et al., 1992). Demonstration of this restriction supports the diagnosis of B-cell lymphoma. They are CD5-, CD10-, CD23- and CD19+, CD20+, CD79a+ (pan-B antigens) CD21+ (follicular dendritic

cells) and CD35+. Bcl2 expression is present only in low-grade and Cyclin D1 is always absent. This phenotype is homologous with that of marginal zone B cells. Indeed in the Revised European-American Lymphoma (R.E.A.L) classification the marginal-zone B cell is recognised as the normal cell counterpart of MALT lymphoma (Harris et al., 1994). Abundance of CD4+ T-cells is often evident in low-grade but not high-grade, supporting the theory that T-cell help is necessary for the initial tumor growth and that the antigen may play a fundamental role.

1.6.5 MALT lymphoma of the stomach and *Helicobacter pylori*

MALT lymphoma of the stomach is preceded by the acquisition of MALT as a result of *H. pylori* infection and several pieces of evidence suggest a link between *H. pylori*-chronic gastritis and the outgrowth of lymphoma (Zucca et al., 1998; Wotherspoon et al., 1991). Infection of the stomach with *H pylori* leads to the accumulation of lymphoid tissue in the gastric mucosa where B cell follicles are characteristically present. Epidemiologic studies report an association between *H. pylori* infection and gastric malignancies, lymphomas (Doglioni et al., 1992; Parsonnet et al., 1994; Vineis et al., 1999) and carcinomas (Parsonnet et al., 1991) (90% of these malignancies are *H. pylori* positive). In vitro neoplastic cells of MALT lymphoma proliferate in the presence *of H. pylori* through the activation of T cells. In one case it was demonstrated that splenic T-cells from the same patient did not respond to the stimulating strain of *H pylori*, demonstrating that the T population responsive to *H. pylori* was local. This explains also why gastric MALT lymphomas in the early phase remain localized. Specimens of gastritis infected by *H. pylori* revealed the presence of the neoplastic B cell clone several years before the

development of lymphoma (Zucca et al., 1998). The progenitor of the neoplastic B cell clone must have some genetic alteration or abnormal biological property that results in its uncontrolled proliferation in the presence of T-cell help. Finally regression of MALT lymphoma after antibiotic eradication of *H. pylori* has been reported in a high number of cases by several independent groups (Montalban et al., 1997; Steinbach et al., 1999). Antibiotic therapy appears to be effective only in low-grade disease restricted to the mucosa or submucosa.

1.6.6 Genetic evidence for a role of the antigen in the development of MALT lymphomas

Immunoglobulin genes expressed by MALT lymphomas show extensive SHMs with evidence in some cases of intraclonal variation. This strongly suggests that direct antigen stimulation plays a role in clonal expansion of the tumor (Du et al., 1996) which is well in keeping with the presence of infectious or autoimmune processes. Further evidence for antigen driven selection is the usage of unusual immunoglobulin V-D-J rearrangements to generate antibody diversity and antigen-binding affinity. In two patients the third complementarity determining region (CDR3) amino acidic sequence was identical, suggesting the presence of a common selecting antigen (Bertoni et al., 1997). The dependence on the antigen and on the local immune response explains the tendency of MALT lymphoma to remain localized, although cases trasforming into aggressive disseminating forms have been described (Zucca et al., 1998). Antigenic stimulation gives a growth advantage to the B cell clone that can respond and that can become predominant and more susceptible to genetic alterations resulting in trasformation and progression (Ghia and Nadler, 1997).

1.6.7 Genetic features of MALT lymphomas

The most common chromosomal translocation is the **t(11;18)(q21;q21)** present in a third of cases (Auer et al., 1997). Recently the genes at the translocation breakpoints were identified as the apoptosis inhibitor gene API2 on ch.11 and MALT1 on ch.18. In this translocation, a fusion gene is created which encodes a chimeric protein consisting of the N-terminal portion of API2 linked to the C terminus of the novel protein MALT1. MALT1 contains a caspase-like domain in the C terminus but no physiologic function has been ascribed to this protein.

A second non-random translocation more rarely detected is the **t(1;14)(p22;q32)** which results in the transfer of the entire Bcl10 gene to chromosome 14. In this location Bcl10 is stimulated by the strong IgH enhancer (Willis et al., 1999). Bcl10 and MALT1 form a tight complex which serves to oligomerize and activate the caspase-like domain of MALT1. This appears to activate the downstream IkB kinase complex (IKK) leading to the induction of NF-kB. Also the API2-MALT1 fusion protein activates NF-kb in the same way, showing dependence upon the same downstream signaling factors. Therefore a model has been proposed where both products of independent translocations activate a common downstream signaling pathway that originates with the activation of the caspase-like domain.

Trisomy 3 was previously reported as a very frequent abnormality in MALT lymphomas, but more recent studies revealed a much lower incidence(Wotherspoon et al., 1995; Ott et al., 1998; Hoeve et al., 1999). **Bcl1 and Bcl2** are not rearranged in MALT lymphomas (Wotherspoon et al., 1990; Clark et al., 1992) and **Bcl6** only rarely (3/34 cases) (Dierlamm et al., 1997). **c-myc** is commonly mutated in the exon I/Intron I regulatory region (7/54 cases) (Peng et al., 1997). **Microsatellite instability** was reported in about 50% of cases (Peng et al., 1996) although other

groups were not able to confirm these data (Sol Mateo M. et al., 1998; Xu et al., 1998; Furlan et al., 1999; Hoeve et al., 1999). **p53** shows loss of heterozygosity in 7% of LG and 29% of HG MALT lymphomas while mutations in p53 were observed in 19% of LG and 33% of HG (Du et al., 1995; Neumeister et al., 1997; Martinez-Delgado et al., 1997).

Studies of tumor-derived rearranged **V_H genes** demonstrated the presence of somatic mutations with evidence of intraclonal diversification, i.e. non-common mutations, in some cases (Qin et al., 1997; Qin et al., 1995).

1.6.8 Model for the pathogenesis of gastric MALT lymphoma

It is likely that some of the above described chromosomal abnormalities provide a selective growth advantage to malignant cells. However, a possible explanation for the pathogenesis of gastric MALT lymphoma may be the following. T cells are activated in the gastric mucosa by *H. pylori* and cytokines and cause a secondary proliferation of B cells. These neoplastic B cells produce antibodies specific for autoantigens and need help from T cells to proliferate. This help is apparently mediated by CD40 and CD40L interactions (Hussell et al., 1996; Greiner et al., 1997). This dependence on T cells help may explain the tendency of LG MALT lymphoma to remain localized and to regress after *H. pylori* eradication. Possibly genetic alterations continue until autonomous growth is reached and later transformation to HG lymphoma can occur. The mechanism of progression from *H. pylori* infection to LG and HG lymphoma is still unclear since for example most patients with *H. pylori* gastritis do not develop lymphoma; therefore additional

environmental, microbial and host genetic factors play a role in lymphomagenesis (Go, 1997; Covacci et al., 1999).

1.6.9 Diagnosis, staging and therapy

The most common symptoms of LG-MALT lymphoma are nonspecific dyspepsia and epigastric pain caused by nonspecific gastritis or peptic ulcer. A few patients present with elevated lactate dehydrogenase (LDH) or β2-microglobulin levels. The best staging system is still controversial but numerous groups adopt the revised version of the Blackledge staging system, recommended by an international workshop held in Lugano in 1993. Unlike most LG B-cell lymphomas of peripheral lymph nodes LG MALT is usually very indolent and often remains localized for prolonged periods; in some cases no progression is seen during several years without treatment. Systemic dissemination and bone marrow involvement occur rarely (Cogliatti et al., 1991; Montalban et al., 1995).

It is difficult to determine what is the best therapy for MALT lymphomas because of the insufficient staging of older reports and more recent studies have been based on retrospective series. Eradication of *H pylori* with antibiotics can be effectively employed as the sole initial treatment (Montalban et al., 1997; Steinbach et al., 1999; Sackmann et al., 1997; Weston et al., 1999; Savio et al., 1996; Nobre-Leitao et al., 1998). Patients which do not respond to *H. pylori* eradication may have harboured HG lesions that initially had not been recognized (Neubauer et al., 1997). This would support the hypothesis of a clonal evolution from a LG MALT still dependent on T-cell help, to an autonomous LG MALT and eventually to an HG MALT, neither requiring antigenic drive for survival and growth. The use of antibiotics as first-line

therapy may postpone the necessity for surgical resection in most patients, and a strict endoscopic followup is therefore recommended (Zucca and Cavalli, 1996). In case of unsuccessful *H. pylori* eradication, triple or quadruple therapy of proton pump inhibitor plus antibiotics should be attempted (Hunt, 1997; Malfertheiner et al., 1997). Patients presenting deep infiltration of the gastric wall or nodal involvement should receive chemotherapy or radiotherapy in addition to antibiotic therapy (Steinbach et al., 1999). No treatment guidelines exist for the management of patients after antibiotic therapy and nonpublished randomized studies may help in this decision. Finally, if surgery is chosen, a total gastrectomy may offer greater chances of cure even though this operation may impair the patient's quality of life.

1.7 B cell Chronic Lymphocytic Leukemia (B-CLL)

B-CLL is the most common leukemia in the Western world and it is characterized by the accumulation of monoclonal B cells resembling small mature lymphocytes with a typical immunophenotype. B-CLL has been extensively studied as model to explain the cellular properties of normal B cells although its cells are so distinctive that the analogies are inappropriate. First, B-CLL is the only blood malignancy not associated with exposure to toxic drugs and chemicals (Hamblin and Oscier, 1998). Second, it is the best example of a human malignancy that involves defects in the induction of programmed cell death. Indeed the gene setting is optimally organized to avoid apoptosis. Last, B-CLL patients have a high prevalence of autoimmune phenomena (autoAb production) causing cytopenias and hypogammaglobulinemia (Kipps and Carson, 1993) (Rozman and Montserrat, 1995).

1.7.1 Phenotype of malignant B-CLL cells

The immunophenotype of malignant B cells closely resembles that of mantle zone lymphocytes of secondary lymphoid follicles (Caligaris-Cappio and Janossy, 1985). They express most of the antigens characteristic of mature B cells and they coexpress CD5 and faint monoclonal surface immunoglobulins (sIgs). These sIgs are usually sIgM+/- sIgD and only rarely sIgG or sIgA (Hashimoto et al., 1995) that often have polyreactive autoAb activity (Boumsell et al., 1978; Ternynck et al., 1974). Surface Igs frequently behave as a rheumatoid factor (Preud'homme and Seligmann, 1972; Borche et al., 1990; Sthoeger et al., 1989). Indeed there is evidence of overusage of V1-69 in germline configuration that codes for monoclonal rheumatoid factor. This faint sIg/CD5 coexpression serves to distinguish B-CLL from other small cell lymphomas (Harris et al., 1994) and provides a clue about the cell of origin. Low levels of sIgM are seen only in normal B cells anergized by interaction with self antigens (Goodnow et al., 1988) and normal CD5+ B cells of the mantle zone often produce polyreactive, low affinity autoAbs (Caligaris-Cappio et al., 1982; Casali and Notkins, 1989). Therefore these similarities have generated the hypothesis that B-CLL is a malignancy of mantle zone anergic self-reactive CD5+ B cells devoted to the production of polyreactive natural autoAbs (Caligaris-Cappio, 1996). The ability to produce autoAbs may provide a survival advantage for the leukemic cells because the interaction of autoreactive sIgs with their self Ags has been shown to prevent the apoptosis of the malignant cell. Atypical cases represent deviations from the orthodoxy of a single entity indicating that B-CLL includes different forms not yet fully identified.

Most circulating B-CLL lymphocytes are in the G_0 phase of the cell cycle (Andreeff et al., 1980), and because of the abnormalities of the BCR are insensitive to

mitogenic signals that induce the proliferation of normal B cells (Nilsson, 1992). The BCR is a complex formed by the sIg and the Iga/Igb (CD79a/CD79b) heterodimer that translates the Ig engagement into the biochemical signals that drive the B-cell response (Reth, 1992). Different abnormalities of BCR determine its low expression on B-CLL cells which may account for the defective signal transduction via the BCR and the reduced induction of protein tyrosine kinase activity (Lankester et al., 1995). Other molecules which normally transduce signals in B cells for example, CD22 induced by anti Ig and CD21 induced by EBV, are weakly expressed on B-CLL cells (Clark and Lane, 1991; Matutes et al., 1994; Rickinson et al., 1982). Eventually B-CLL cells have a marked reduction of functional Na+/H+ antiporter units which respond to LPS proliferative stimuli (Ghigo et al., 1991). Note that B-CLL cells produce many cytokines but none of these is individually able to overcome the G_0 blockade. Their role in the natural history of B-CLL is still unclear.

1.7.2 Molecular abnormalities

It has been extremely difficult to define the molecular abnormalities in B-CLL for two reasons: the very low mitotic rate and the usage of mitogens that stimulated T cells and not B cells. An abnormal karyotype is observed in approximately 50% of patients (Juliusson et al., 1990). The most frequent abnormalities are deletions or translocations of ch. 13q14 (Fitchett et al., 1987), trisomy 12 (Han et al., 1984) and deletions of ch. 11q23 (Dohner et al., 1997) and 6q (Offit et al., 1993). About half of the patients have a single abnormality and the remainder have two abnormalities or a more complex pattern.

The most frequent abnormality is that of chromosome **13q14** and patients with it have a benign disease and survive as long as their matched controls (Oscier et al., 1990). In contrast, **trisomy 12** is associated with progressive disease (Oscier et al., 1997) and atypical cellular morphology (Matutes et al., 1996). Trisomy 12 determines MDM2 gene amplification and overexpression with inactivation of p53. Moreover trisomy 12 is associated with unmutated VH genes, whereas CLLs with 13q14 deletions show heavy somatic mutations (Oscier et al., 1997). Deletions at **11q23** are found in up to 10% of CLLs and patients with this abnormality are reported to have extensive lymphnode involvement and require an aggressive clinical course (Dohner et al., 1997). Mutations or deletions of p53 at **17p13** occur in 15% of patients and are associated with advanced disease, high proliferation rate and shortened survival (Lens et al., 1997). **t(14;18)** is rare (Dyer et al., 1994) but **Bcl2** is overexpressed (Pezzella et al., 1990; Schena et al., 1992) probably because of hypomethylation causing increased RNA transcription (Hanada et al., 1993). B-CLL cells express high levels of Bcl2, BclxL and Bax (Gottardi et al., 1996). Bax is a partner for Bcl2 and Bcl-xL synergizes with Bcl2. In B-CLL there is no **Fas** expression (Mapara et al., 1993) and no **c-myc** expression (Larsson et al., 1987), both proapoptotic molecules. This pattern of expression favours the suppression of apoptosis. Bcl3 translocations, **t(14;19),** are rarer than t(14;18) and in 50% of cases are associated with trisomy 12 (Michaux et al., 1997).

1.7.3 Immune deficiency and autoimmunity in B-CLL

Immune incompetence is a cardinal feature of B-CLL. At the time of diagnosis most B-CLL patients show expansion of the T cell mass and normal Ig levels, particularly

in an early stage of disease. After having received treatment they show deficiency of T cell function with inversion of the CD4 to CD8 ratio and low immunoglobulin levels (Bartik et al., 1998; Chapel, 1987). B-CLL cells secrete TGF-ß, which is a potent inhibitor of B-cell proliferation (Lotz et al., 1994) , and release high levels of circulating IL-2 receptor (Semenzato et al., 1987), which might act as a sponge for endogenous IL-2 and thus down-regulate the T helper function. Furthermore, B-CLL cells fail to present soluble antigen and alloantigen (Dazzi et al., 1995; Eris et al., 1994), whereas normal, activated B cells are very effective APCs (Lanzavecchia, 1985). The inability of B-CLL cells (and of anergic normal B cells) to properly act as APCs is explained, at least in part, by the low levels of sIg and the suboptimal expression of the costimulatory molecules CD80 and CD86. The defective expression of CD79b may be an additional element. It should also be considered that CLL CD4+ T cells express CD40 ligand (CD154) mRNA but fail to express the molecule on the cell surface after CD3 ligation. CD40+ leukemic cells have been shown to down-modulate CD154 on the surface of normal, donor-activated CD4+ T cells, provided that the ratio between CD4+ normal T cells and leukemic B cells declines below a critical level (Cantwell et al., 1997). In a disease characterized by an excess of CD40+ leukemic cells, such a receptor-mediated down-modulation of CD154 could interfere with the ability of normal T cells to interact with normal B cells or other APCs. Moreover given the role of CD154 in T cell induction of isotype switching this defect in CD154 may account for the deficiency of CLL patients to produce IgG of various subclasses (van de Velde et al., 1991). The conclusion to be drawn from all these data is that the accumulating malignant B-cell population per se is a hurdle obstructing the production of normal antibody and may lead to progressive immunoincompetence.

Any explanation of the immunodeficiency has to be consistent with the fact that up to 25% of patients develop autoimmune syndromes like hemolytic anemia, thrombocytopenia, and, more rarely, neutropenia or pure red cell aplasia (Hamblin et al., 1986). In the vast majority of cases, the autoAbs are polyclonal and, by definition, not produced by the malignant clone (Kipps and Carson, 1993). The production of polyclonal, monoreactive, high-affinity, pathogenic autoAbs of IgG class in the context of a malignancy characterized by the accumulation of anergic B cells that produce monoclonal, polyreactive, low-affinity autoreactive IgM antibodies is an obvious paradox. Two other facts need to be accommodated by any global theory. First, autoimmunity in B-CLL is almost entirely confined to an attack on the formed elements of the blood (Kipps and Carson, 1993; Hamblin et al., 1986). Second, autoimmunity is much more common in patients treated with fludarabine, a drug known to induce profound suppression of circulating CD4+ T cells (Myint et al., 1995).

It is not unreasonable to suppose that immune incompetence and autoimmunity are two sides of the same coin. A common explanation of these immune defects may lie in aberrations of the immunoregulatory circuits that involve the malignant B cells as well as the T cells and residual normal B cells. One of these abnormalities may well be the role of B-CLL cells as APCs. In vitro experiments have shown that stimulation of CD40 on the membrane of B-CLL cells (as well as on anergic normal B cells) by the T cell-expressed CD154 may up-regulate the surface expression of CD80 and CD86 molecules and thus transform anergic CD80-CD86- cells into efficient CD80+CD86+ APCs (Eris et al., 1994; Ranheim and Kipps, 1993). The role of malignant B cells as APCs is underscored by experiments with severe combined immunodeficiency mice: when reconstituted with peripheral blood lymphocytes from

CLL patients (Kobayashi et al., 1992), they frequently develop polyclonal human IgG autoAbs to human redblood cells even though the CLL itself does not proliferate within the mouse. Further complexity is added to the system by the recent report that CLL malignant B cells can express CD154 and may demonstrate a T cell-type costimulatory capacity that provides inappropriate B-cell "help"(Schattner et al., 1998).

Another eccentricity may lie in the Ig gene usage of the CLL cells. In patients with autoimmune hemolytic anemia, it has been suggested that a particular CDR3 region is used by the leukemic cells (Efremov et al., 1996). This implies that the antibody used may beinvolved in the pathogenesis of the hemolytic anemia, perhaps by promoting an increased clearance of senescent enterythrocytes.

Finally, T-cell numbers are increased in B-CLL patients (Kay, 1981) and have profound abnormalities of their antigen receptor (TCR) repertoire (Serrano et al., 1997). If we assume that certain T-cell subsets are able to prevent the development of autoreactive B cells, it follows that when these T-cell subsets are absent, eg, after treatment with purine analogs, autoreactive B-cell clones may easily emerge and expand. B-CLL cells may become activated by T helper cells (perhaps via CD154 if an adequate T cell to malignant B cell ratio is maintained) in an environment such as the spleen, where senescent anucleate blood cells are removed. Such activated malignant B cells may then become able to present red cell and/or platelet membrane degradation products (self-Ag) and drive residual normal B cells to produce target-restricted pathogenic autoAbs (Caligaris-Cappio, 1996). This process would be facilitated if the B-CLL cells used certain Ig genes and might become clinically evident when the control exerted by T cells becomes deficient.

Finally, constitutive expression of FasL on B-CLL cells, inducing apoptosis on T cells, is proposed as a mechanism for decreased absolute T cell count and inverted CD4 to CD8 ratio.

1.7.4 Immunoglobulin genes: "switch" and "variable" region configuration

As previously reported, B-CLL cells express low-density surface immunoglobulins, most often of IgM or IgM/IgD isotype. Only a small proportion of B-CLL is characterized by the expression of isotype-switched immunoglobulins (Keating, 1999; Hashimoto et al., 1995).

Rearrangements in the immunoglobulin Sμ regions without productive isotype switching have been observed in B-CLL cases. Crossen *et al.* found in 31% of B-CLL rearrangements of the Cμ–switch region that could not be explained by normal rearrangements of the immunoglobulin genes. The molecular nature and the clinical implications of these alterations were not investigated (Crossen and Morrison, 1998). Studies by Hakim *et al.* detected rearrangements of the J_H-Cμ intron in about 40% of B-CLL cases but only one rearranged fragment was cloned revealing a 300 bp insertion in the Sμ region (Hakim et al., 1993).

In addition to rearrangements of the switch regions, B-CLL $sIgM^+$ cells also show a capacity to undergo isotype switching *in vitro* when stimulated through surface CD40 in the presence of IL10 (Malisan et al., 1996). Moreover studies by Fais *et al.* reported the presence of Cα and Cγ transcripts bearing the same V_H region of the IgM in 50% of IgM^+CLL patients (Fais et al., 1996) and studies by Efremov *et al.* reported the presence of Cα and Cγ transcripts in all analyzed cases even though only 2% of the population expresses sIgG on FACS analysis (Efremov et al., 1996).

Under physiological conditions, isotype switching occurs in temporal correlation with somatic mutation of the variable genes in the germinal center microenvironment (Gearhart et al., 1981; Siekevitz et al., 1987; Siekevitz et al., 1987) and the recent discovery of AID (Activation-Induced Cytidine Deaminase) (Kinoshita and Honjo, 2001) has revealed an unexpected link between isotype switch recombination and somatic hypermutation. This does not imply that a mutated B-CLL is also isotype-switched or vice versa. Indeed, these somatic processes can occur independently, and there is evidence suggesting that variable gene somatic mutation already occurs in IgM of the late primary response before isotype switch (van Es et al., 1992), and that isotype switching can occur in the absence of somatic mutations (Wysocki et al., 1992; Gilmore et al., 1987; Gilmore et al., 1987).

Traditionally B-CLL has been viewed as a tumor of "naïve" B cells possibly arising in the follicular mantle zone (Caligaris-Cappio, 1996). However, some B-CLL with somatically mutated immunoglobulin variable region genes have been described (Schroeder and Dighiero, 1994). Since somatic mutation is believed to occur in germinal centers (Berek and Milstein, 1987), the presence of such mutations indicates that the cell of origin has passed through the germinal center. Thus, it has been suggested that B-CLL comprises two different disease subtypes, one arising from a "naïve" B cell, the other from a "memory" B cell. Patients with non-mutated variable genes have a distinctly more malignant disease and a much shorter survival than those with somatic mutations (Maloum et al., 2000; Hamblin et al., 1999).

1.7.5 Staging systems and prognostic factors

The Rai and Binet staging systems have provided the backbone of clinical staging (Rai et al., 1975) (Binet et al., 1981). Rai system divides B-CLL cases in 5 stages (0, I, II, III, IV) while Binet in 3 stages (A, B, C). Both systems consider the following parameters: lymphocytosis, lymphadenopathy, hepato or splenomegaly, hemoglobin and platelets. It is usually accepted that patients with Binet stage C and Rai stages III and IV should be treated at the onset. The median survival of these patients is 3 to 4 years. Earlier stage patients with low tumor burden usually have a delay of therapy. Some of these are classified as patients with "smoldering CLL" who have the same survival as matched controls (Montserrat et al., 1988; Anonymous1990).

Important prognostic factors for survival are age, atypical lymphocyte morphology, cytogenetic abnormalities and doubling time of the lymphocyets (Zwiebel and Cheson, 1998). In addition patients with a diffuse BM infiltration have a worse prognosis (Rozman et al., 1984). Soluble CD23 and β2 microglobulin are also important factors for prognosis (Sarfati et al., 1996) and patients with leukemia cells with high telomerase activity have a shorter median survival than those with low activity.

1.7.6 Therapy for B-CLL

Chemotherapy. Chlorambucil has been used for decades to treat patients with advanced stage disease although it does not appear to improve survival (Dighiero et al., 1998). Leukemic cells of treated patients generally develop resistance to further therapy. In vitro studies demostrated that this resistance is due to increased protein

substrate phophorylation and increased Ku-DNA binding activity (Muller et al., 1998).

Fludarabine has a response rate of 78% and a median survival of 63 months when given to patients with aggresive disease (Keating et al., 1998). Treatment can result in immune suppression, causing a decrease in blood CD4+ and CD8+ T cell levels and an increased incidence of infections (Schilling and Vadhan-Raj, 1990).

Autologous stem cell transplantation carries the risk of contamination with CLL cells (Gahn et al., 1997; Gribben et al., 1994). A few studies have shown complete clinical response (Dreger et al., 1998; Pavletic et al., 1998; Sutton et al., 1998). Allogenic stem cell transplantation is limited to younger patients who take advantage from graft-versus-leukemia response.

Biologic therapy. Rituximab is a humanized monoclonal antibody specific for CD20 which can induce responses in half of the patients with relapsed follicular lymphoma, possibly by directing Ab-dependent cytotoxicity against CD20+ lymphoma B cells (Reff et al., 1994; Maloney et al., 1997; McLaughlin et al., 1998). However CLL B cells express lower levels of CD20 than most lymphomas and several clinical trials in B-CLL are ongoing. CAMPATH-1H is a humanized mAb specific for human CD52, a molecule present on CLL B cells and most lymphocytes, including T cells. One study reported clinical responses in half of the treated patients even if associated with increased incidence of infections (Osterborg et al., 1997).

Gene therapy. A tumor vaccine with CLL B cells infected with an adenoviral vector expressing CD154 (CD40L) has been planned. It should induce CLL B cells to become highly effective APC and induce autologous T cells to generate CTL specific for non-infected leukemia cells.

1.8 Aim of thesis

The study of the immunoglobulin heavy chain (IgH) locus located on chromosome 14q32 in hematologic tumors has allowed the identification of new important genes and established the maturation of precursor-cells. To this end two regions of the IgH locus have been investigated: the V(D)J segments, encoding for variable regions (V) of the produced antibody and the switch regions, indispensable for isotype or class switching from IgM/D to IgG, IgE, IgA.

The aim of the present thesis is the investigation of IgH switch regions and variable regions in two B-cell tumors displaying mainly IgM isotype, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and B-cell chronic lymphocytic leukemia (B-CLL).

Discovery of new genes has led to the definition of their role in normal cells and to their use for diagnosis and, in some cases, for therapy. Numerous hematologic tumors are nowadays quite easily diagnosed through identification of specific alterations of the IgH locus, such as chromosome translocations generating fusion proteins with new or defective roles. For this reason identification of alterations in the IgH locus is fundamental to the definition of pathogenetic events and eventually of possible diagnosis systems and new therapeutic options. Chromosome translocations may involve the V(D)J or the switch regions since V(D)J recombination and isotype switching are two events occurring in all B cells at different points in the development. For example, translocations of c-myc, bcl-1 and bcl-2 into the J_H region in endemic Burkitt's lymphoma, mantle zone lymphoma and indolent lymphoma respectively, occur during V(D)J recombination. Translocations

of c-myc, bcl-3 and bcl-6 into switch regions in sporadic Burkitt's lymphoma, CLL and diffuse lymphoma occur during isotype switch recombination. Chromosome translocations are not the only alteration that may occur in the IgH locus during the B-cell development. There might be deletions, insertions, inversions which might have a strong impact on the cell's development and behaviour.

To define the maturation of precursor-cells the region of great interest is the V(D)J region. This region is first amplified and sequenced and then compared to the deposited germline V regions to identify somatic hypermutations. These hypermutations contribute to affinity maturation of the antibody and may be of two types, common mutations or non-common mutations. In the first case all clones of a given V gene show the same mutation, in the second case each clone shows its own specific mutation generating intraclonal diversity. The presence of intraclonal diversity is a hallmark of germinal center B cells, the presence of common mutation of post germinal center B cells.

Because of all the important information one can obtain studying the IgH locus (identification of new genes, of the maturation stage of prescursor-cells and possibly of pathogenetic events) this thesis studied the IgH locus in gastric MALT lymphomas and B-CLL. Some aspects of the IgH locus were already known for these tumors before starting.

MALT lymphoma cells derive from marginal-zone B cells and show V regions with intraclonal diversity. Nobody ever investigated the status of switch regions before this thesis. In gastric MALT lymphoma we looked for the presence and nature of possible rearrangements of the switch regions. To determine whether gastric low-

grade MALT lymphomas with or without aberrant rearrangements in the switch regions differ in B-cell maturation stage, we investigated the V_H gene mutational status. V_H genes were sequenced and analyzed for the presence and nature of somatic mutations (common and non-common) and for the presence of possible antigen selective pressure.

In B-CLL, previous studies of V genes allowed the definition of this tumor as a "heterogeneous disease" since it could be divided into two subgroups. One arising from a "naïve" B cell and carrying no mutations in the V region, the other one arising from a "memory" B cell and carrying mutations in the V genes. It was also established that patients with non-mutated variable genes had a distinctly more malignant disease and a much shorter survival than those with somatic mutations. As far as the switch regions in B-CLL, is concerned some studies had detected the presence of anomalous switch rearrangements in a proportion of cases but the molecular nature of these rearrangements was never defined, nor their relation with clinical course (Hamblin et al., 1999). In this thesis the presence and the molecular nature of the switch region rearrangements in 38 cases of B-CLL was investigated. Because of the association of V genes mutational status with clinical course and because of the link of isotype switch with somatic mutations, we investigated the relation of switch region rearrangements to the clinical course.

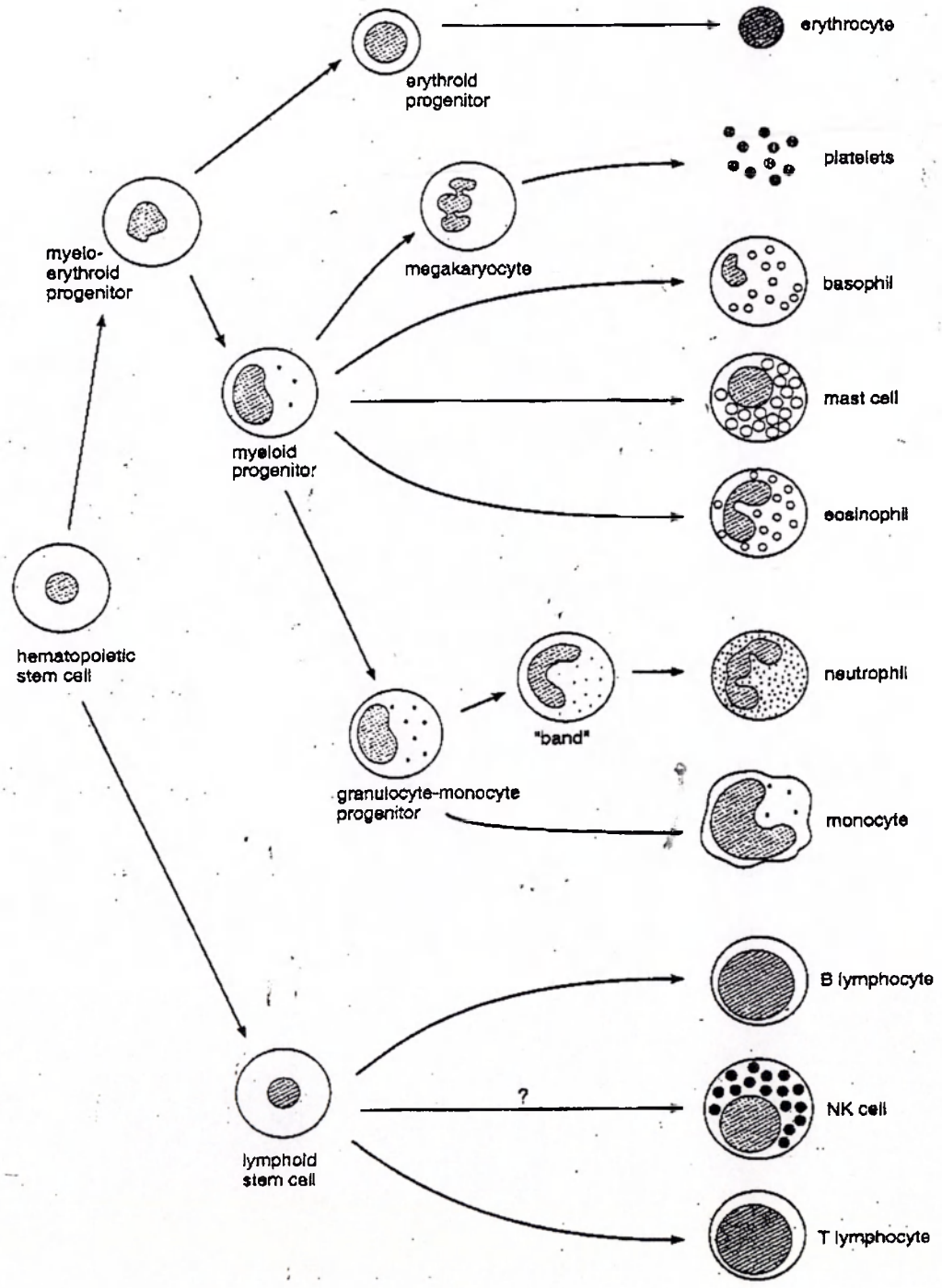


Figure 1.1

Schematic overview of hematopoiesis showing the erytroid, myeloid and lymphoid pathways

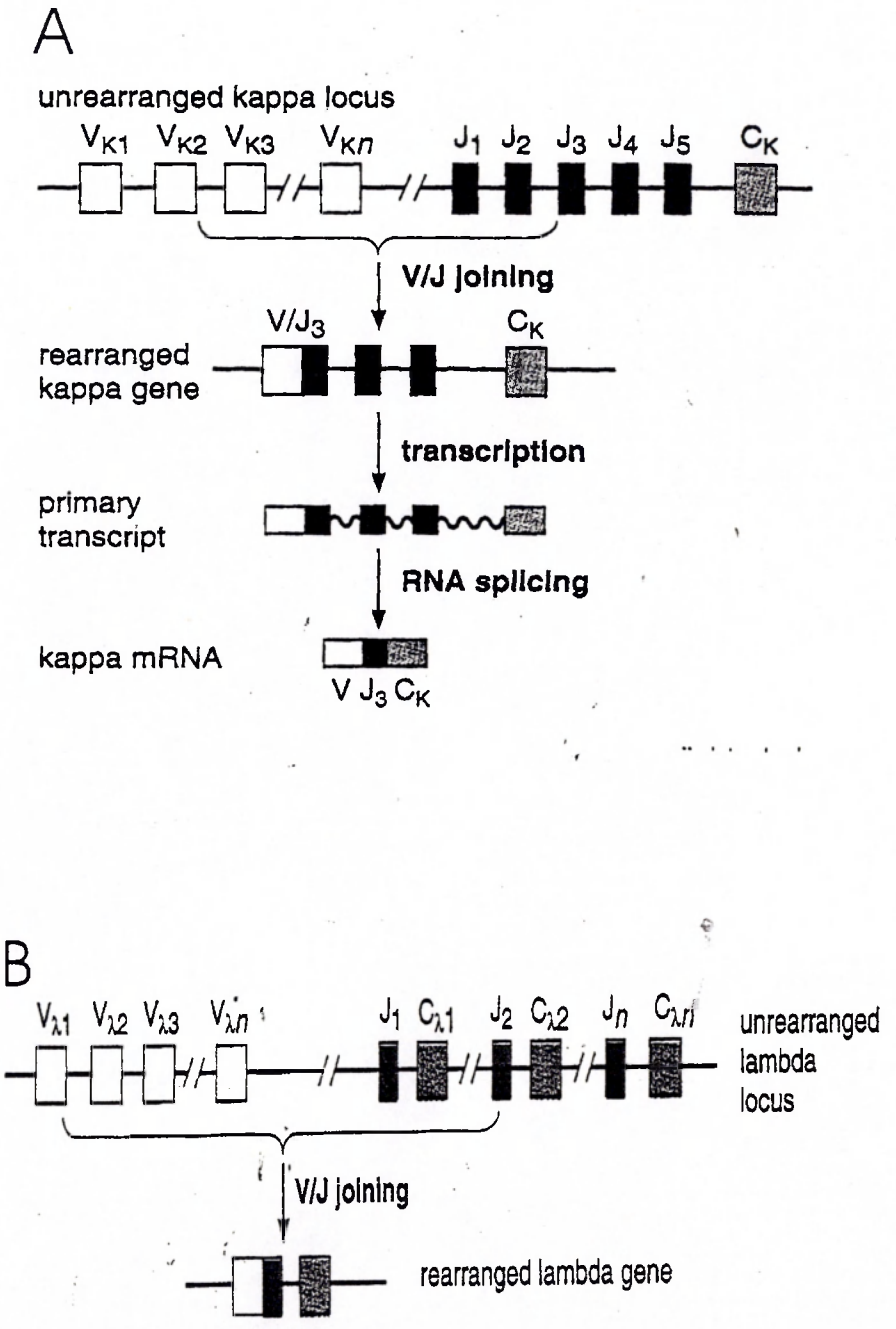


Figure 1.2

Configuration of the Kappa light chain locus on chromosome 2 (A) and of the Lambda light chain locus on chromosome 22 (B): assembly and expression of the light chains

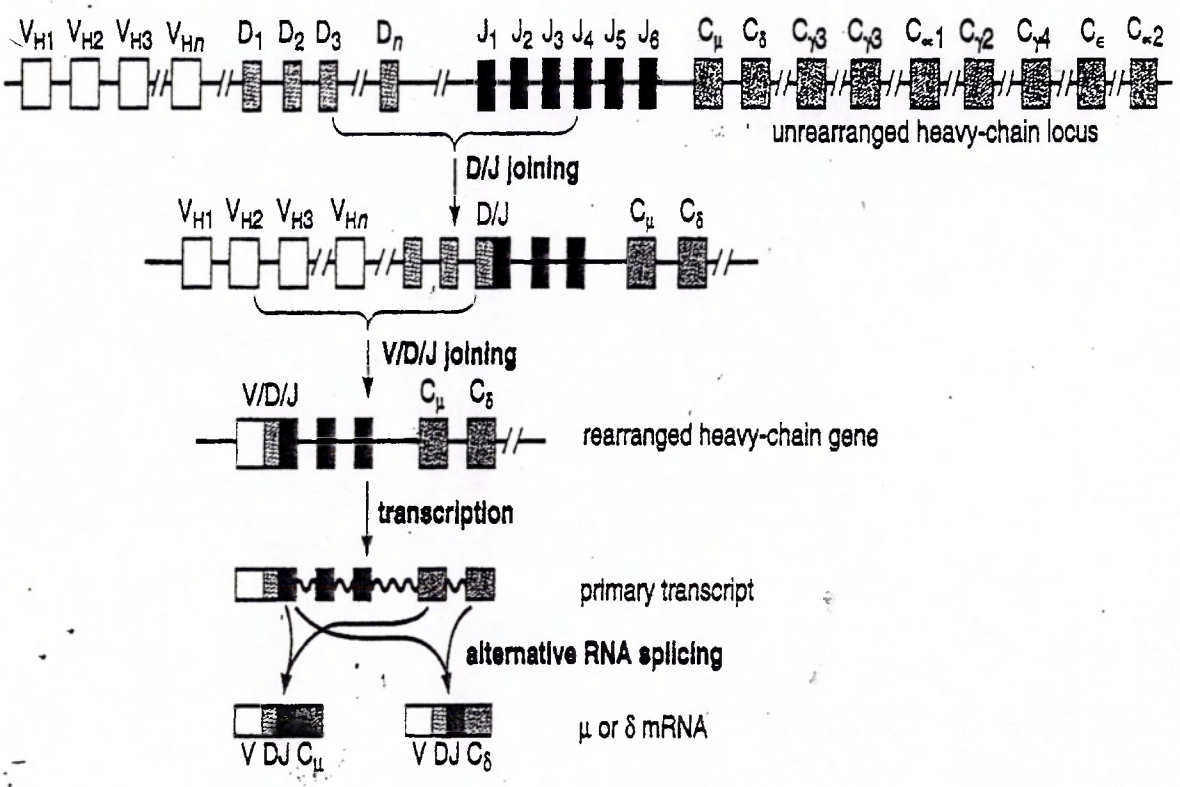


Figure 1.3

Rearrangement and expression of the IgH locus on chromosome 14q32

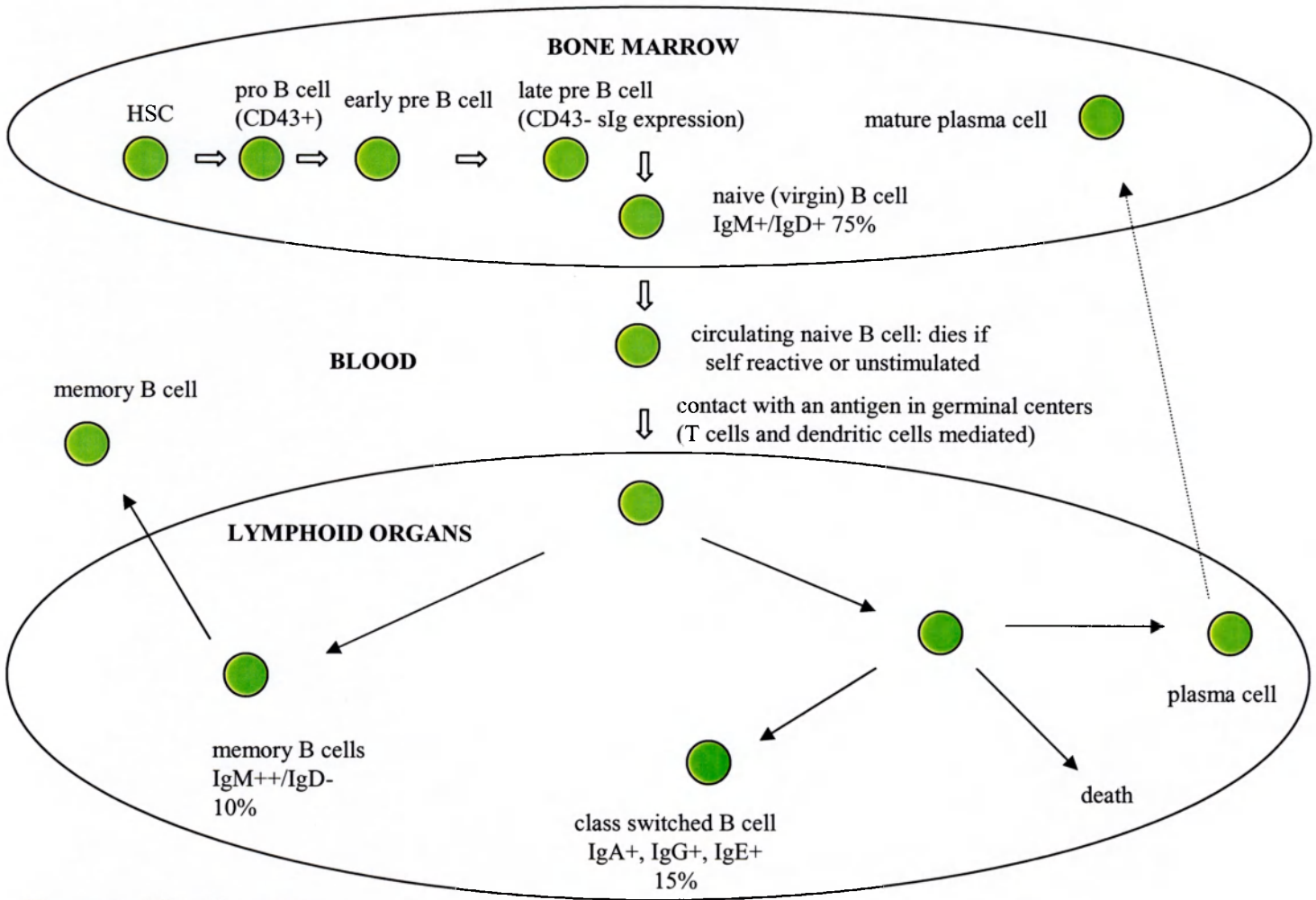


Figure 1.4 B-cell ontogeny

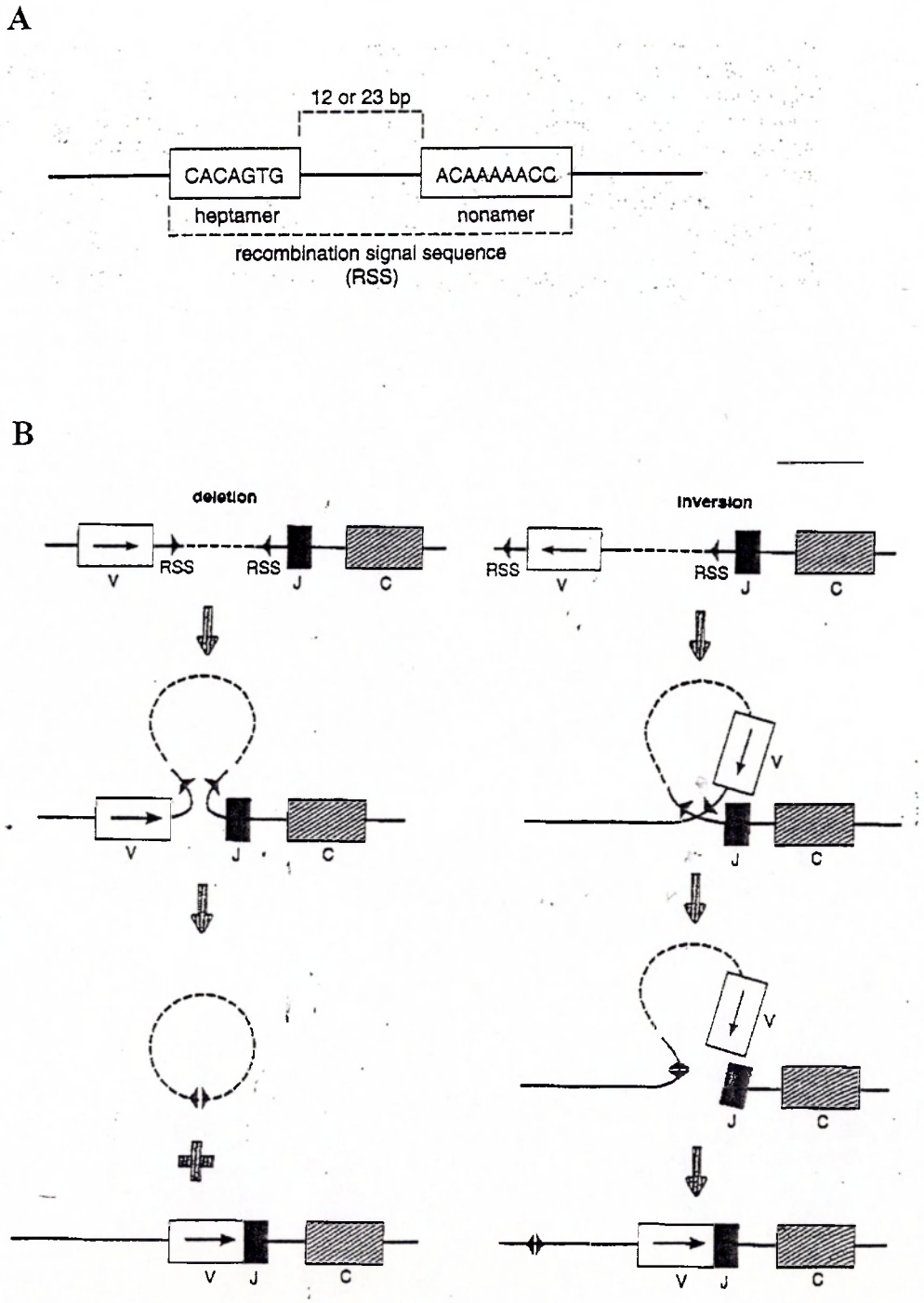


Figure 1.5

Mechanism of immunoglobulin K gene rearrangement. **A.** RSS marks all sites of recombinase action in heavy and light chain genes. **B.** The chromosome undergoes either deletion (left) or inversion(right) depending on the original orientation of the V segment with respect to the J and C segments.

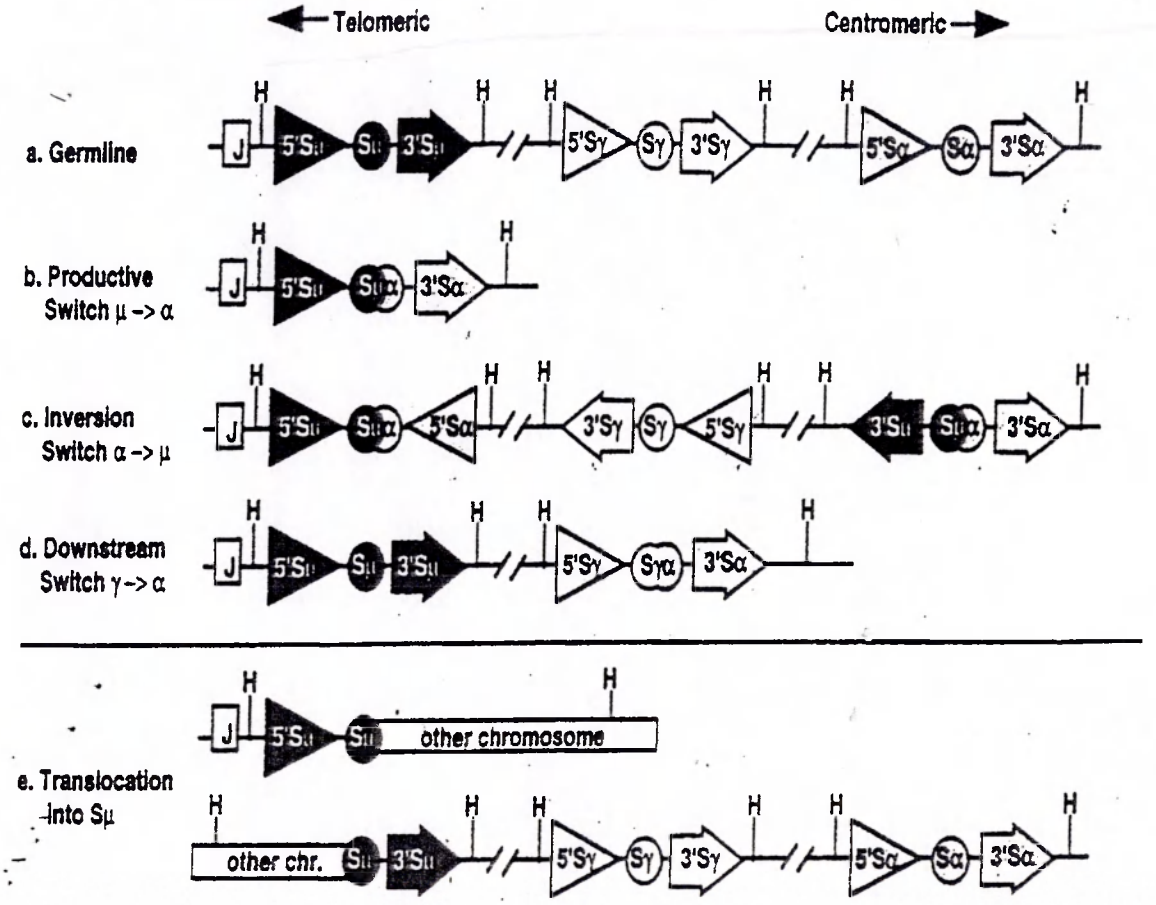


Figure 1.6

Schematic map showing IgH switch recombination events and an hypothetical translocation into Sμ region

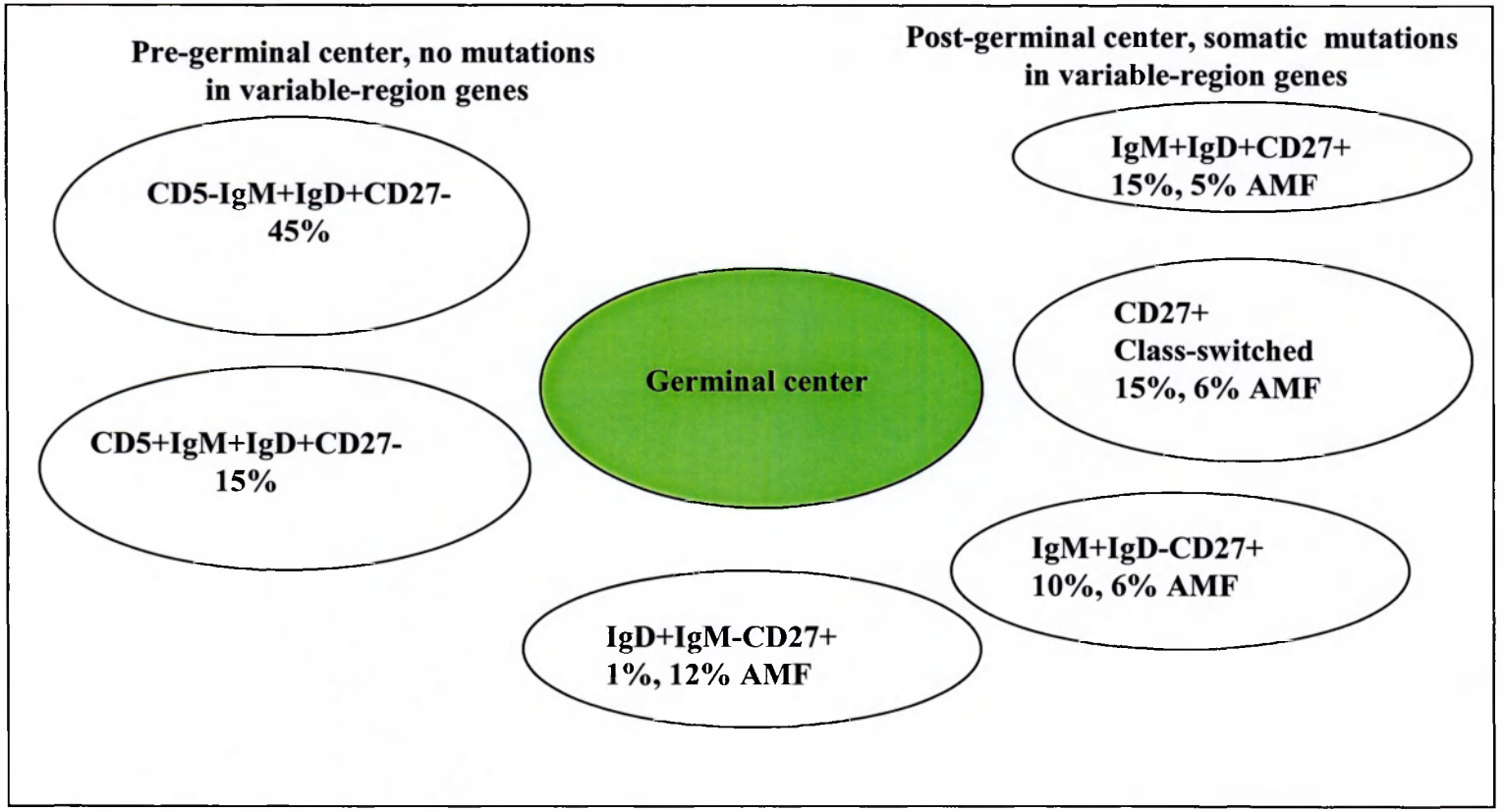


Figure 1.7
Cellular composition of the peripheral blood B-cell repertoire in humans
AMF= average mutation frequency of variable-region genes

Chapter 2

MATERIALS AND METHODS

2.1 General procedures

All solutions employed for the preparation and manipulation of nucleic acids were made up using distilled water and autoclaved before use or filter-sterilized using a 0,22 μm filter. Unless stated otherwise, all chemical reagents were supplied by Sigma (St.Louis, MO) or Merck (Darmstadt, Germany) and all enzymes were purchased from New England BioLabs (Hitchin, Hertfordshire, UK) or Boehringer (Mannhem, Germany).

2.2 Flow cytometry (FACS)

Flow cytometry was used to detect the expression of cell surface molecules. $5x10^5$ cells were washed twice in ice-cold PBS and then resuspended in PBS-0.03% BSA. The cell suspension was incubated at 4°C for 45 min with the specific fluorochrome-conjugated monoclonal antibody (at the optimal pre-determined concentration) or with fluorochrome-conjugated polyclonal antibody, as specified in Results. As a negative control, PBS-0.03% BSA was added in place of the antibody. After washing twice with PBS and spinning down (1200 rpm for 5 min), the cells were resuspended in PBS and analyzed using a FACScan or a FACScalibur flow cytometer.(Becton

Dickinson, Mountain View, CA). Unless stated, all mAbs were purchased from Pharmingen (San Diego, CA) and diluted as indicated in the supplier's sheet.

2.3 Immunohistochemistry

Immunoperoxidase assays were carried out on paraffin-embedded sections of MALT lymphoma gastrectomies. Briefly, 1 to 2 μm consecutive sections of formalin-fixed, paraffin-embedded tissue were cut and mounted in poly-L-lysine (Sigma, St.Louis, MO) coated-slides, deparaffined in xylene and rehydrated in grade alcohol. Endogenous peroxidase activity was blocked by treatment with 0.3% hydrogen peroxide in methanol for 30 min. Slides were incubated overnight with different biotinylated monoclonal antibodies specific for Ig isotypes (DAKO). Antibody reactivity was detected by incubation with streptavidin-conjugated horseradish peroxidase (DAKO), and peroxidase activity was detected by amino-ethyl carbazole (AEC). Staining without antibody was performed as a negative control.

2.4 Genomic DNA extraction

High-molecular weight DNA extraction was performed on selected MALT lymphoma specimens and on B-CLL peripheral blood mononuclear cells (PBMCs) following a "salting out" method. Samples were lysed by incubating overnight at 56° C with agitation in 3 ml of lysis buffer (0.01M TrisHCl pH 8.2, 0.4M NaCl, 0.002M Na_2EDTA), adding fresh 0.5% SDS and 0.5 ml proteinase K solution (1 mg proteinase K in 0.5 ml 1% SDS, 0.002M Na_2EDTA). The following day, 1 ml of 6M NaCl was added to the samples, shaken for few seconds and centrifuged at 2500 rpm

for 15 min. The supernatant was then transferred to a new tube and two volumes of 95% ethanol was added. After nucleic acid precipitation, the pellet was washed three times in 70% ethanol and finally resuspended in 50-200 µl of TE solution.

2.5 Nucleic acid precipitation

Nucleic acids were precipitated by adding 2.5 volumes of ethanol and 1/10 v/v sodium acetate and chilled at –20°C for 30 min. After incubation samples were centrifuged at 13,000 rpm for 15 min (Sepatech Biofuge, Heraeus Instruments) and pellets were then washed twice with 70% v/v ethanol and air dried before resuspension in distilled water.

2.6 Agarose gel electrophoresis of DNA

Agarose gels were prepared by adding agarose (0.8% to 1.5% w/v) to 1X TAE buffer (Tris-acetate-EDTA) and boiling in a microwave cooker for 5 min. After cooling to below 50°C 1 µl of ethidium bromide stock solution (10mg/ml) was added and gels were poured into a gel former with a well-comb in place. After setting, the gel was submerged in a tank containing 1X TAE buffer. Loading buffer (5X solution: 5ml glycerol, 1ml 10x TAE, 1ml 10% bromophenol blue, 1ml 10% xylene cyanol, 2ml water) was added to the DNA solutions which were then loaded into the wells. Electrophoresis was performed at constant voltage (25 to 100 Volts) for variable periods of time (20 min to overnight). DNA was visualized using a transilluminator with short wave ultraviolet light and DNA fragments were sized by reference to a DNA marker run at the same time.

2.7 Enzymatic digests

Eight or 10 μg of genomic DNA was digested with the appropriate restriction enzyme (50U/reaction) in a 100 μl digestion mix containing appropriate buffer supplied by the manufacturer, BSA (100 μg/ml), spermidine (1 mM) and RNaseA (50 μg/ml).

2.8 Probes

A pair of probes for each isotype, including probes for the non-conventional switch region upstream of δ (5'σμ and 3'σδ) were synthesized by PCR using specific oligonucleotides reported in Table 2.1.

2.9 Southern blotting

Digested genomic DNA was fractionated by gel electrophoresis on a 0.8% agarose gel, denatured with 0.5N NaOH/1.5M NaCl, neutralized with 1M Tris-HCl/1.5M NaCl and transferred overnight to nylon filters with 20X SSC. The following day, filters were air-dried and fixed with UV light (UV Stratalinker).

2.10 Labelling of DNA probes with radio-isotopes

Double-stranded DNA fragments were labelled using mixed hexadeoxyribonucleotide primers of random sequence employing reagents supplied in a kit (Boheringer). 25ng of DNA were boiled at 100°C for 10 min and then mixed in a buffered solution containing dNTPs and primers in a total volume of 20 μl.

Labelling was performed at 37°C for 1 hr with 50 μCi of [α-^{32}P] dCTP using 10 Units of Klenow subunit of DNA polymerase. The mix was then passed down a G-50 Sephadex column to remove unincorporated nucleotides. The labelled product was then boiled for 5 min before being added to the hybridization solution.

2.11 Hybridization conditions

Filters were hybridized overnight at 42°C with 1x10^6 cpm/ml of probe in a hybridization buffer containing 1M NaCl, 50mM Tris-HCl pH 7.4, 40% formamide, 10% dextran sulfate, 1% SDS and 100 ug/ml salmon sperm DNA. The following day, filters were washed sequentially in solutions containing 2X SSC/0.1%SDS (20° C), 1X SSC/0.1%SDS (40°C) and 0.1X SSC/0.1% SDS (65°C). They were finally exposed to autoradiogram films at –80°C or scanned on a Phosphorimager.

2.12 Long-Distance Inverse PCR cloning

Genomic DNA, digested with the appropriate restriction enzyme, was purified using the Wizard DNA Clean-up system (Promega) and 400ng was self ligated at 14°C overnight in a total volume of 500 μl with 5U of T4 DNA ligase (Promega). The ligated DNA was again purified using the Wizard DNA Clean up system and eluted in a final volume of 50 μl. 5μl were used as template for the PCR reaction which contained 200μM dNTPs, primers at 0,2 μM, 1U rTth polymerase (Perkin Elmer) and 1,2mM magnesium acetate in a final volume of 50 μl. The enzyme was added after the first cycle (Hot Start procedure). PCR cycles were as follows: 1 cycle 94°C 3', 30 cycles 94°C 1', 60°C 1', 72°C 6', 1 cycle 72°C 10'. 5μl of the PCR reaction

were reamplified for 30 cycles using identical conditions with nested primers. After PCR amplification, the products were gel purified and cloned using the PCR-Script Cloning kit (Stratagene). Clones were sequenced using an ABI Prism automated sequencer

2.13 Polymerase Chain Reaction (PCR) of V_H regions

The V_HDJ_H region was amplified from genomic DNA (1 μg) using a consensus forward primer, annealing in the framework 1 region [$FR1_c$:AGGTGCAGCTG(GC)(AT)G(GC)(AGT)GG], in combination with a joining heavy chain consensus primer [JH_c:ACCTGAGGAGACGGTGACC(AG)(GT)(GT)GT]. The PCR mixture contained 1X Taq buffer, 0.2 mM of each dNTP, 1 mM of $MgCl_2$, 10% DMSO, 1 U of Taq polymerase (GIBCO) and 1 μM of each primer in a final volume of 50 μl. PCR amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 34 cycles at 94°C for 1 min, at 62°C for 1 min and 72°C for 2 min, with a final extension step at 72°C for 10 min.

Rearranged V_HDJ_H sequences were also amplified using a semi-nested strategy and different primer pairs: FR2 [5' TGG(AG)TCCG(AC)CAG(GC)C(CT)(CT)C(AGCT)GG 3'], annealing in the framework 2 region, and LJH [5' TGAGGAGACGGTGACC 3'], annealing in the joining region. The first round of PCR was performed in 50 μl, containing 200 μM of each dNTP, 4.5 mM $MgCl_2$, 0,7 μM primers and 1X Taq buffer. Hot-start was performed by the addition of 0.25 U of Taq polymerase (GIBCO) after 7 min at 95°C. PCR consisted of 30 cycles at 93°C for 45 s, 50°C for 45 s and 72°C for 1 min

followed by a final extension step at 72°C for 5 min. One microliter of the first amplification was used for second-round PCR under identical conditions, except the VLJH [5' GTGACCAGGGT(AGCT)CCTTGGCCCCAG 3'] primer was substituted for the LJH primer, $MgCl_2$ concentration was decreased to 2.5 mM and 20 cycles were performed.

2.14 PCR to determine clonality

To determine tumor clonality, a semi-nested amplification of the variable region from framework 2 (FR2) to the joining segment (JH) in the IgH locus was performed. Cases that did not show monoclonality by FR2/JH amplification were then PCR amplified from FR1 to the JH region. Resulting PCR products from FR1/FR2 amplification were analyzed on a 6% or 8% acrylamide gel respectively. Single or double PCR bands indicated monoclonality.

2.15 Purification of V_H fragments from gel

Bands corresponding to V_H genes were isolated from a 2% low-melting agarose gel using Jetquick Kit (Genomed) and following the manufacturer's instructions.

2.16 Cloning of V_H PCR products

Purified V_H fragments were cloned using the TOPO TA Cloning Kit (Invitrogen) and following manufacturer's instructions. Both strands of the inserts were sequenced from 3 or more randomly selected bacterial colonies using an ABI sequencer to

obtain the consensus sequence or sequence of the dominant clone (shared by most clones).

2.17 Storage of bacteria

Bacteria were collected during the log phase of growth and were resuspended in a solution containing growth medium and 1/3 v/v glycerol. Storage was performed in cryotubes at –80°C. To resurrect bacteria from a frozen stock they were scraped, inoculated in fresh growth medium and grown overnight in a shaking incubator at 37°C.

2.18 Small scale preparation of plasmid DNA ("miniprep")

Plasmid DNA was prepared from small cultures of bacteria using a Wizard Minipreps System (Promega, Madison,WI), following the protocol supplied by the manufacturer. This procedure is based on the alkaline lysis method for rapid extraction of plasmid DNA from bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein and Gillespie, 1979).

Single colonies were inoculated into 5 ml of L-broth containing ampicillin and incubated overnight in a shaking incubator at 37°C. The next day cells were harvested and resuspended in 200µl of cell resuspension solution. 200µl of cell lysis solution was then added and mixed, followed by 200µl of neutralization solution which causes precipitation of denatured proteins, SDS, cellular debris and chromosomal DNA, due to high salt concentration. After centrifugation, supernatants were mixed with 1 ml of resin and loaded onto a minicolumn. 2 ml of wash solution

was added to remove salt and the DNA was eluted by applying distilled water to the column.

2.19 Automated sequencing of DNA by the chain termination method

Sequencing reactions were performed using an ABI PRISM Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer), according to the manufacturer's instructions. Enzymatic extension was performed in an asymmetric PCR using a single primer, dye-labeled terminators and the AmpliTaq DNA polymerase FS, a mutant form which has no 5'-3' nuclease activity and reduced discrimination for dideoxynucleotides. Each reaction contained: template DNA (500 ng), 3,2 pmol Primer, 6 μl Terminator Mix containing the polymerase FS, A, G, C, T Dye-labeled, terminators dITP, dATP, dCTP, dTTP, $MgCl_2$, pyrophosphatase and water to 20 μl volume. Extension was performed following the cycles: 96° C for 4 min, 25 cycles at 96° C for 30 sec, 50° C for 20 sec 60° C for 4 min and a final cycle at 60° C for 3 min. After precipitation of DNA, samples were loaded onto a polyacrylamide gel and analyzed on an ABI PRISM 377 DNA Sequencer which was operated by the Sequencing Service of Istituto Nazionale Tumori, Milan.

2.20 Statistical analyses

Statistical analyses were performed using a GraphPad Prism computer program (GraphPad Software, Becton Dickinson). To analyze B-CLL cases, the Fisher's Exact Test was used, for MALT lymphoma cases the Student's t test. Statistical significance was considered to be $p < 0,05$.

2.21 PCR of Sμ regions and sequence analysis

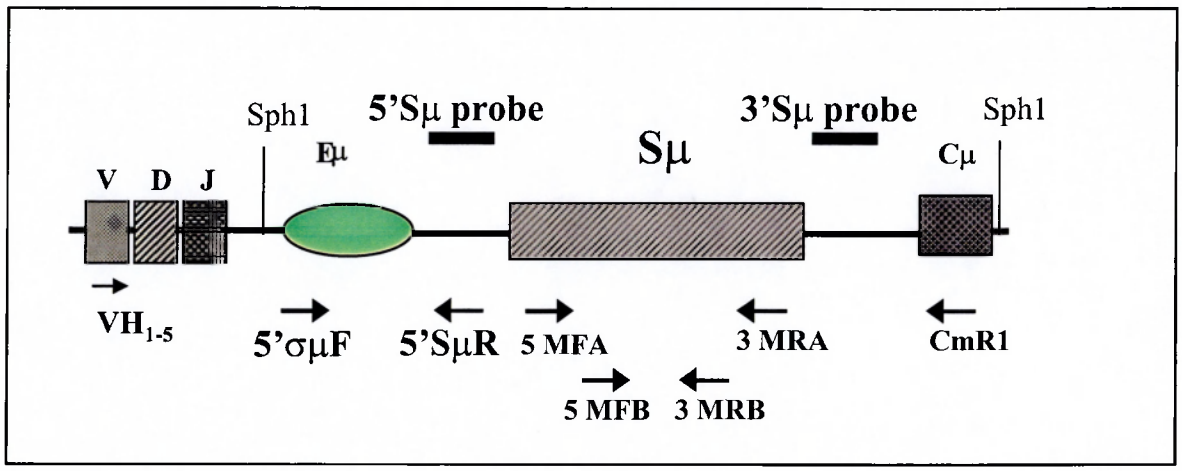
Genomic DNA (100 or 300 ng) was used as template in PCR reactions with several oligonucleotides (Figure 2.1). The reaction mixture contained dNTPs at 200 μM, primers at 0.2 μM, 1 U rTth polymerase (Perkin Elmer) and Mg acetate at 1.2mM, in a final volume of 25 or 50 μl. PCR cycles were: 1 cycle at 94°C for 3 min, 34 cycles at 94°C for 1 min, 62°C for 1 min and 70°C for 5 min and a final step at 70°C for 10 min. The enzyme was added after the first cycle (Hot Start procedure).

PCR-amplified Sμ regions were aligned to the germline sequences HSIMMDL-HSJHCMU using the BLAST database to identify deletions and/or DNA rearrangements.

2.22 PCR from the V_HDJ_H to the Sμ region to localize Sμ deletions

Genomic DNA (100 ng) of each patient with Sμ rearrangements was amplified using a "touchdown" strategy. Forward primers annealing to the specific V_H family expressed by each case were used in combination with a reverse primer complementary to the 3' Sμ region (3MRA or 3MRB). PCR reaction was performed in a final volume of 100 ul with 20 pmol of each primer, 200 μM of dNTPs, 1,2 mM of Mg acetate and 2U of rTth DNA polymerase (PE Applied Biosystem) with Hot Start technique. Amplification consisted of an initial denaturation step of 1 min at 94°C followed by 24 cycles at 94°C for 15 sec and 68°C for 10 min with an increasing time of 15 sec in the last 10 cycles. The final extension step was performed at 72°C for 10 min.

A



B

PRIMER	SEQUENCE 5'-3'	GENBANK	LOCALIZATION
5'σμF	CAGATCTGAAAGTGCTCTACTG	90885-90906	HSIMMDL
5'SμR	TCAGCTAAAGCCATCTCATTGCC	92520-92498	HSIMMDL
5MFA	GGCTCCTAAATTCTTGGTCTCA	92291-92312	HSIMMDL
5MFB	GGCAATGAGATGGCTTTAGCTGA	92498-92520	HSIMMDL
3MRB	GTGATGGGGAACGCAGTGTAGA	3803-3781	HSJHCMU
3MRA	CCGCAGGCAGCCAATAGAGT	3931-3911	HSJHCMU
CmR1	ACACGTGTCAGCCCGGTGCC	534-515	HSJHCMUDE

Figure 2.1

A. Localization of the switch μ (Sμ) probes used for Southern blot assay and of the primers used for PCR amplification of IgH Sμ regions. **B.** Primer sequences and relative position in GenBank database

Table 2.1 Sequence of PCR primers used to amplify switch region probes

Probe	Forward primer	Reverse primer
5'σμ	CAGATCTGAAAGTGCTCTACTG	CTTATCCTAAAGTGAGTAGTTG
3'σδ	CACCGAAACCTCTGGAGGGAAG	TGTGCTGGACCACGCATTTG
5'Sμ	TTTGAAGGAGAGGTCGCACGAG	TCAGCTAAAGCCATCTCATTGCC
3'Sμ	TCTACACTGCGTTCCCCATCAC	CGTTCTGAGTGCCCTCACTACTTG
5'Sγ	CAGAATGGTCATAATCGCTGCC	CATCCCGTCATGTTCCTCGTG
3'Sγ	GCTATTCCAAGACAGGGGGTTCC	CAGAAAGCTTGCAGGACCG
5'Sα	CAGCATCCAGCCACATCTG	AGATCCTTCCTGCCTGGTTAG
3'Sα	CATGGTGCAGGAGCTGTGTAAC	TCCACTCTGGTGTGAGTGAAGG
5'Sε	AAGAGAACCTCCCCAGCACTC	GTCGGGTCTCTGACTTCTTGGTCT
3'Sε	CGGGGCTGGATACTGTGATTTTG	TATCATCAGGCTGGGCTCAGGAAG

The sequence of the primers is given from 5' to 3'

Chapter 3

Analysis of the immunoglobulin switch regions in gastric MALT lymphomas

Frozen and paraffin-embedded tissue samples obtained from MALT lymphoma gastrectomies were retrieved from our Department of Pathology. For each tumor sample, a Giemsa-stained paraffin section was histopathologically analyzed and classified either as low or high-grade according to the criteria described by Isaacson and Norton (Isaacsoon and Norton, 1994). Five low-grade and two high-grade gastric MALT lymphomas were analyzed for the isotype and for clonality. The isotype of surface immunoglobulins was determined immunohistochemically on paraffin sections using monoclonal antibodies specific for human Ig isotypes and clonality was determined by PCR.

Five cases were found to be IgM producing and two cases non-producing. All cases showed monoclonality by PCR amplification from FR1, FR2 to JH (Table 3.1). Specimens of MALT lymphoma chosen to study rearrangements within the IgH switch regions had a percentage of lymphoid tumor cells ranging from 40% to 80%.

3.1 Strategy to analyze immunoglobulin switch regions

To study rearrangements within the IgH switch regions, five pairs of probes (one pair for each isotype) that hybridize upstream (5') and downstream (3') of each switch

region (Fig.3.1) were synthesized by PCR. Using these probes and restriction enzymes with digestion sites outside the pair of switch probes, it was possible to classify by Southern blot each switch region as germline, or having undergone either a physiological or aberrant isotype switch recombination. Upstream and downstream switch probes detect the same restriction fragment for germline switch regions. Physiological switch recombinations between any two switch regions result in the generation of a restriction fragment detected by two probes from different switch regions. In contrast, aberrant switch recombination generates restriction fragments uniquely detected by only one switch probe.

Genomic DNA from the seven cases of gastric MALT lymphoma was digested with different restriction enzymes and screened by Southern blotting using the set of probes specific for the switch regions.

3.2 Two cases of low-grade MALT lymphoma have aberrant switch μ rearrangements.

Case n° 1 produced Ig of the M isotype and showed a small rearranged 5'Sμ fragment (3.0 kb) on a HindIII digest that did not cohybridize with any 3'Sμ fragment (Fig. 3.2b). On a SphI digest, the 5'Sμ probe hybridized to an 8.5 kb aberrant fragment which was also identified by the 3'Sμ probe. One possible explanation for the presence of a 3'Sμ aberrant fragment on a SphI but not on a HindIII digest could be that the aberrant HindIII digest fragment is either too big or too small to be seen by Southern blot. This patient sample showed two rearranged bands (12.0 kb and 4.8 kb) with the 5'σμ probe on a BglII digest while the 3'σδ probe only detected the germline fragment. No rearranged fragment cohybridized

with any of the fragments identified by downstream probes, which only identified germline fragments (data not shown). Considering the hybridization pattern of the HindIII and SphI blots , it appears that in this case only one IgH allele underwent aberrant recombination in the Sμ region, since only one rearranged fragment is present in each lane. On the contrary, the BglII hybridization pattern suggests that the aberrant switch recombination involved both IgH alleles (or that the breakpoint split the probe). It is possible to suppose that a "downstream" recombination starting at σδ deleted on one allele sequences downstream of σδ but maintained a germline configuration upstream of σδ. In this situation the 5'Sμ and the 3'Sμ probes would be able to detect only one rearranged allele and the δ probes would detect the rearrangements on both alleles. Rearrangements downstream of the Cμ-Cδ genes were recently reported for IgM expressing Follicular Lymphoma (FL) and it was suggested they may reflect a tumor-specific deregulation of the class-switch machinery (Vaandragen et al., 1998).

Case n° 2 was positive for IgM production after staining with monoclonal anti-isotype antibodies. Genomic DNA from this case was extracted, digested with 3 restriction enzymes (HindIII, SphI and BglII) and hybridized with the switch probes (Fig.3.2a). The 5'Sμ probe showed the germline fragment and two rearranged fragments of 7.5 kb and 6.4 kb on HindIII digest and of 4.7 kb and 3.0 kb on SphI. The 3'Sμ probe hybridized to the germline fragment only. As far as the δ region is concerned, the 5'σμ probe identified the germline fragment and two rearranged fragments of 7.0 kb and 3.8 kb on a BglII digest while the 3'σδ probe hybridized to the germline fragment only. All rearranged fragments were aberrant since they did not hybridize with any other switch region probe (data not shown).

3.2.1 Amplification and cloning of the larger aberrant 5'Sμ fragment in Case n° 2

To amplify the 4.7 kb rearranged fragment observed on SphI digest with the 5'Sμ, genomic DNA from case n° 2 was digested with SphI, self-ligated and long distance inverse-PCR was performed using four primers (5MFA, 5MRA, 5MFB, 5MRB), internal to the 5'Sμ region (Table 3.2). A first PCR amplification was done using primers 5MFA and 5MRA and a second nested amplification using 5MFB and 5MRB. As expected, two bands were obtained, one of 4.5 kb and the other of 2.8 kb which correspond to to the 4.7 kb and 3.0 kb bands identified by Southern blot and therefore to the two Sμ alleles. The two bands were purified by gel extraction, cloned using the PCR-Script Cloning kit (Stratagene) and fully sequenced. The sequencing of the 4.5 kb band revealed a deletion starting from the Sμ region at nucleotide 628 in HSJHCMU (GenBank nucleotide sequence of the IgH Sμ region) to just upstream of Cμ exon 1 (Cμ exon 1 is from 145-456, Cμ exon 2 is from 547-882, Cμ exon 3 is from 1128-1445) at nucleotide 124 in HSIGCMUDE (GenBank nucleotide sequence of the human IgH Cμ and Cδ genes) (Fig. 3.3). The sequence continued through Cμ exon 2 and it was once again interrupted upstream of Cμ exon 3 (position 1018 in HSIGCMUDE) with γ 3 sequences upstream of the two membrane exons starting at 10541 in D78345 (GenBank nucleotide sequence of the human Ig γ heavy chain) and ending at the SphI site at position 10933. Regions upstream of Sμ to the SphI site at 90430 in HSIMMDL (GenBank DNA sequence of the human immunoglobulin D segment locus) are in the germline configuration. If we assume that the IgH region downstream of the SphI site at 10933 in D78345 is in germline configuration, it is plausible that the 6,4 kb fragment on the HindIII blot and the 7.0 kb fragment on the BglII blot correspond to the sequenced fragment.

3.2.2 Identification of the human switch alpha 2 region from the cloning of the 3.0 kb aberrant fragment in Case n° 2

The characterization of the 3.0 kb band hybridized by the 5'Sμ probe on a SphI digest in case 2 allowed the identification of the human switch alpha 2 region. The complete sequence of the 2.8 kb fragment obtained by long-distance inverse PCR was compared with the GenBank database using the BLASTN program and it was found that the aberrant fragment was generated by a switch recombination between Sμ and switch alpha (Sα) (Fig. 3.4). Switch alpha regions are located in the IgH locus upstream of the two alpha constant genes (Cα1 and Cα2), analogous to the switch regions and constant genes of other isotypes. Cα2 constant region exists in two allotypic forms α2m(1) and α2m(2) (Chintalacharuvu et al., 1994).

In the 2.8 kb aberrant fragment, the sequence upstream of Sμ, which normally contains the IgH enhancer (Eμ), is in germline configuration while the Sμ region recombined with the Sα region and resulted in the formation of a Sμ/Sα hybrid region. The Sα sequence (GenBank accession number AF239920) from the first nucleotide to nucleotide 144 showed 95% identity with the deposited GenBank Sα1 sequence (HUMIGACHSR from 2544 to 2687), while from nucleotide 189 to 692 the degree of identity with Sα1 decreased to 91%. From nucleotide 693 to 876 the sequence was 99% identical to the constant alpha 2 region (Cα2) allotype 2 (sequence S71043). The only difference occurred at nucleotide 853 where an adenine was replaced by a guanine resulting in an amino acid exchange (Glu to Ala) at position 21 in the constant alpha CH1 domain. Since the repetitive alpha region is localized in front of the Cα2 and it shows 98% identity (from nucleotide 509 to nucleotide 797) with the *Pan troglodytes* Ig alpha 2 heavy chain switch region, it

probably represents a new sequence of the human Sα2 region. Indeed, to our knowledge only a short part of the human Sα2 sequence (HUMIGHATC), localized upstream of the sequenced region, has been deposited in the GenBank.

3.3 One case of low-grade MALT lymphoma undergoes an aberrant recombination resulting in a partial deletion of the Sμ region.

In the IgM producing **case n°3** (Fig. 3.5), the 5'Sμ and 3'Sμ probes cohybridized to the germline fragment and to a fragment of 8.0 kb on a HindIII digest and of 7.6 kb on a SphI digest. 5'σμ and 3'σδ probes cohybridized to the germline and to a 15.8 kb fragment on a BglII digest.

To define the nature of the smaller rearranged bands the Sμ region was amplified from tumoral genomic DNA by PCR using primers 5'MFB and 3'MRB (Table 3.2). As expected, a smaller band compared to the germline was amplified (1200bp versus 3557bp in HSJHCMU), indicating a deletion of 2350bp internal to the Sμ region. The 1200bp fragment was cloned into a PCR-Script vector and sequenced. Two regions, from nucleotide 246 to 521 and from 3300 to 3803 in HSJHCMU, were in germline configuration, indicating that the breakpoint occured somewhere between the two sequenced germline regions.

3.4 Two cases of low-grade MALT lymphoma did not show switch rearrangements.

Cases n° 4 and n°5 showed a hybridization pattern identical to that of placental DNA (data not shown) with all switch regions in germline configuration.

3.5 The two cases of high-grade MALT lymphoma had rearrangements in Sμ and one of them also in downstream switch regions.

Case n°6 was diagnosed as high-grade MALT lymphoma and it was positive for IgM production (Table 3.1). On a SphI digest it showed, a 5'Sμ rearranged fragment of 6.0 kb and a 3'Sμ rearranged fragment of 6.5 kb in addition to the germline fragment (Fig. 3.6a). Furthermore, on a BglII digest, the 5'σμ and 3'σδ probes respectively hybridized to a 4.5 kb and a 5.0 kb rearranged fragment. Downstream switch probes showed no rearrangements.

This aberrant switch recombination may represent a chromosomal translocation involving the Sμ region. The 5'Sμ probe would hybridize to the telomeric 14q32 which moved to an unknown chromosome and the 3'Sμ probe would hybridize to the der (14q32). This aberant fragment has not been cloned yet.

Case n°7 (Fig. 3.6b) did not produce Igs and showed multiple rearranged switch fragments on a HindIII digest: one 3'Sμ fragment of 18.0 kb, one 3'Sα fragment of 9.5 kb and three 5'Sγ fragments of 6.8 kb, 6.5 kb and 3.0 kb, only two of which (6.8 kb, 6.5 kb) cohybridized with 3'Sγ fragments.

3.5.1 Amplification and cloning of the aberrant 3'Sμ fragment in Case 7

Since the aberrant 3'Sμ fragment was very large (18 kb), additional Southern blots with numerous restriction enzymes were performed. In this way, a rearranged 3'Sμ fragment of 5.0 kb was identified on an EcoRI digest, which was a good candidate to be cloned. This fragment was amplified by long distance inverse-PCR

using primers (3MFA, 3MRA, 3MFB, 3MRB) (Table 3.2) in the 3'Sμ region. A 4.5 kb band was obtained which was cloned and sequenced.

The sequence showed a V(3-43)/D/JH(3) rearrangement followed by a deletion of the IgH intronic enhancer (Eμ) starting from position 90367 in HSIMMDL (downstream of JH6) to the Sμ region (2291 in HSJHCMU) (Fig. 3.7a). Moreover, there was an insertion of a 336bp region from the gene KIAA0307 (5983-6319) into the Sμ region with subsequent deletion of the Sμ sequence from position 2962 to 3216 in HSJHCMU. Sequences of the 3 breakpoints are reported in Fig. 3.7b.

To confirm the IgH enhancer deletion, we performed a PCR on tumor and placenta genomic DNA with a forward primer in the JH region (JHF) and a reverse primer (3MRB) in the Sμ region (Table 3.2). This PCR produced a 3.0 kb band in the tumor versus a 6.0 kb product in placenta. As far as the three Sγ physiological switch fragments (6.8 kb, 6.5 kb and 3.0 kb) is concerned, it is likely that these are extra Cγ polymorphic alleles. The 3.0 kb band that was identified only with the 5'Sγ probe but not with 3'Sγ probe could be an extra polymorphic Cγ allele in which HindIII cuts within the 5'Sγ probe, thus giving rise to two germline bands.

Table 3.1

Description of MALT lymphomas analyzed for immunoglobulin switch regions configuration

Case N°	Diagnosis	Sex	Age (yr)	Ig Isotype	Clonality
1	Low grade	M	47	M	MON
2	Low grade	F	59	M	MON
3	Low grade	F	73	M	MON
4	Low grade	M	53	M	MON
5	Low grade	M	65	NP	MON
6	High grade	F	31	M	MON
7	High grade	F	59	NP	MON

Abbreviations: NP=non-producing, MON=monoclonal

Table 3.2

Oligonucleotides used for long-distance inverse PCR cloning

Name	Sequence 5'-3'	Position
5MFA	GGC TCC TAA ATT CTT GGT CTC A	92291-92312 HSIMMDL
5MRA	TCC CTC TAG ATA ACA GTC ATC A	92140-92118 HSIMMDL
5MFB	GGC AAT GAG ATG GCT TTA GCT GA	92498-92520 HSIMMDL
5MRB	CTC GTG CGA CCT CTC CTT CAA A	91910-91888 HSIMMDL
3MFA	CAG TCA GGC CTC AGA GTG CA	4095-4115 HSJHCMU
3MRA	CCG CAG GCA GCC AAT AGA GT	3931-3911 HSJHCMU
3MFB	CAA GTA GTG AGG GCA CTC AGA ACG	4353-4377 HSJHCMU
3MRB	GTG ATG GGG AAC GCA GTG TAG A	3803-3781 HSJHCMU
JHF	TCT CCT GCC AAG AGA GCC CC	88884-88903 HSIMMDL

HSIMMDL: GenBank sequence of the human immunoglobulin D segment locus

HSJHCMU: GenBank sequence of the human IgM heavy chain switch region.

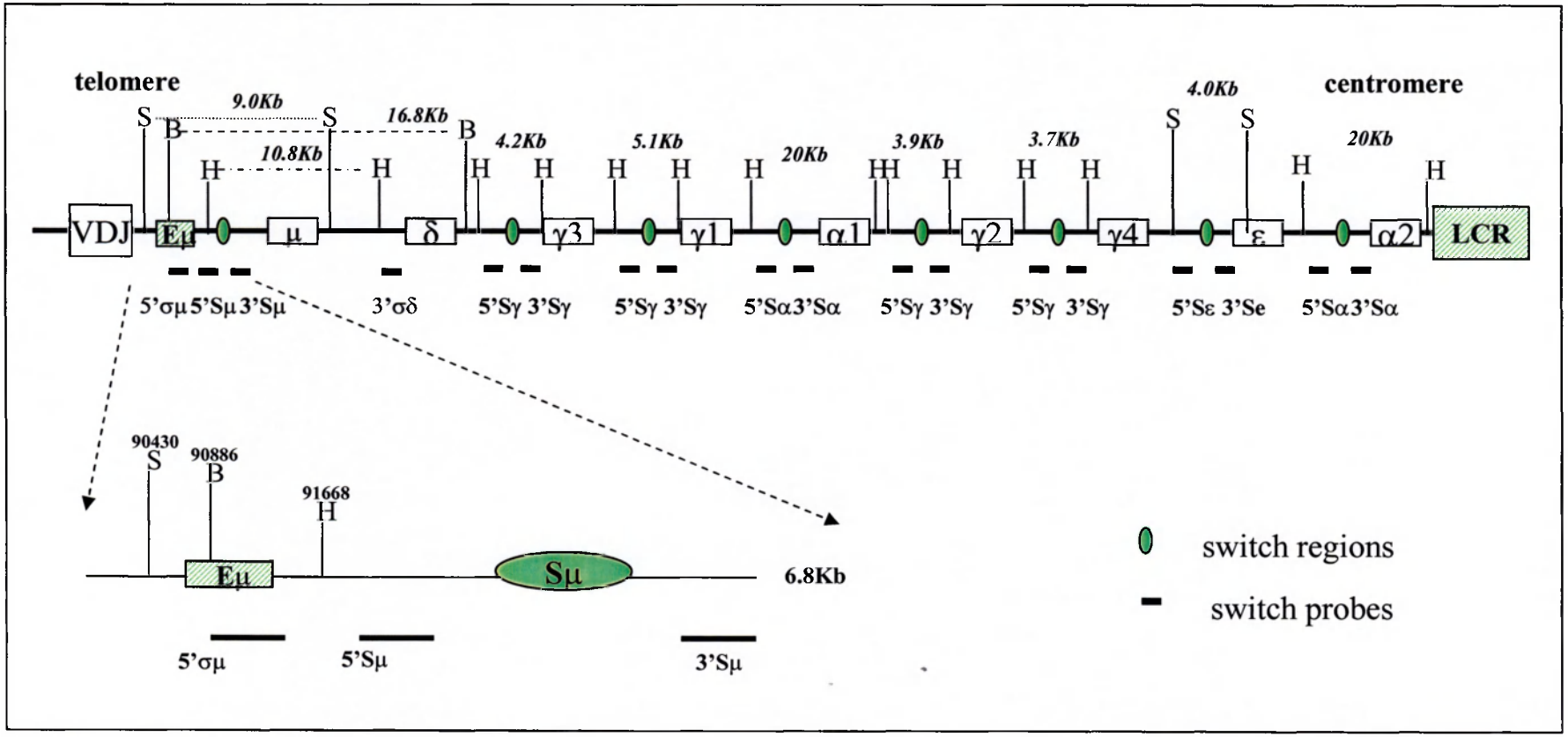


Figure 3.1

Upper: IgH locus organization showing the localization of the switch probes and of the restriction enzymes (vertical lines) used for the Southern blot assay. Since there is no conventional switch region upstream of Cδ the 5'σμ and 3'σδ probes were used to detect δ rearrangements. Distances between restriction sites in germline configuration are also represented. H=HindIII, B=BglII, S=SphI, LCR=Locus Control Region.

Lower: enlargement of the intronic enhancer region (Eμ). Numbers above the restriction sites indicate their postions in the GenBank sequence HSIMMDL

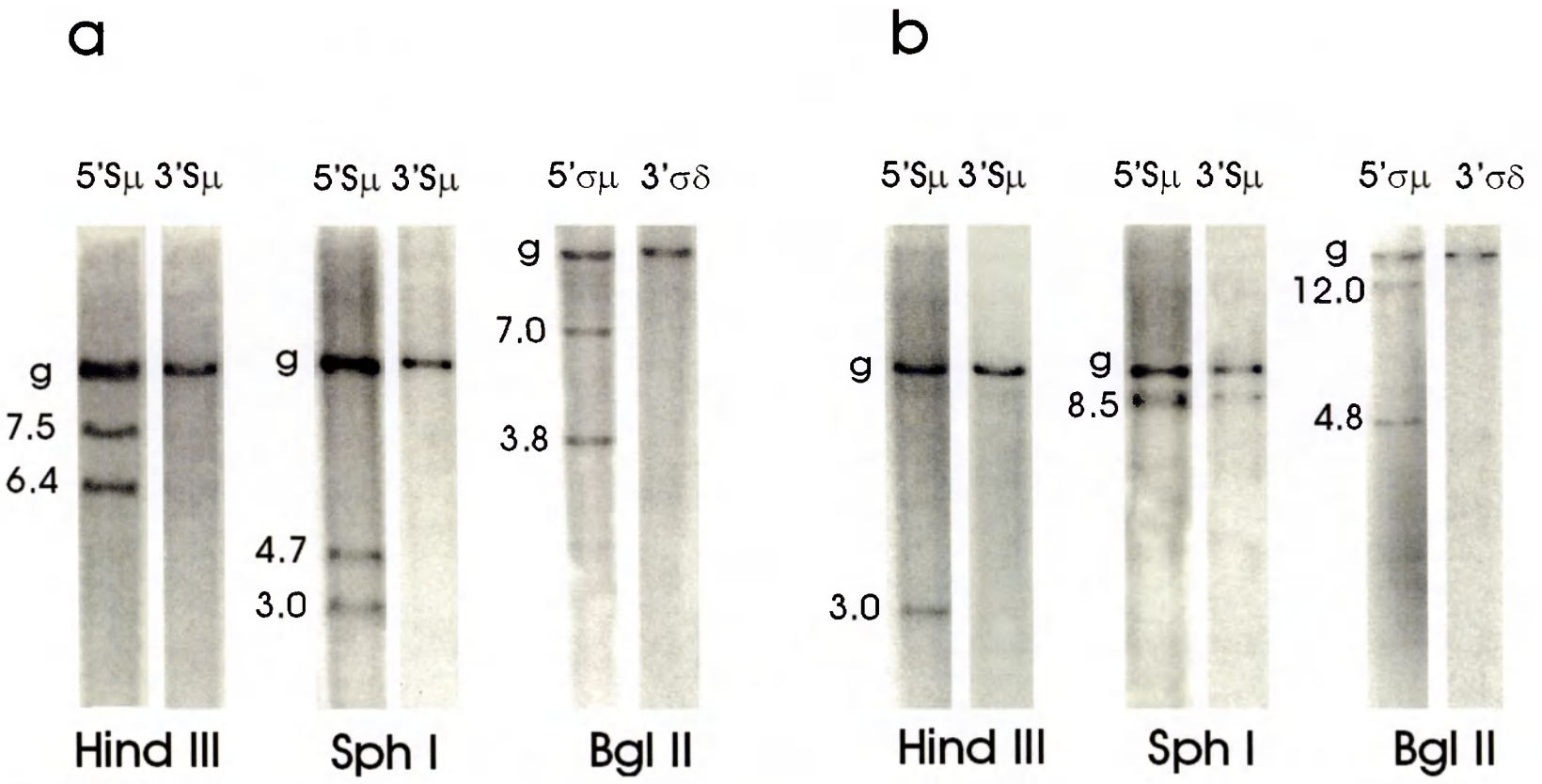
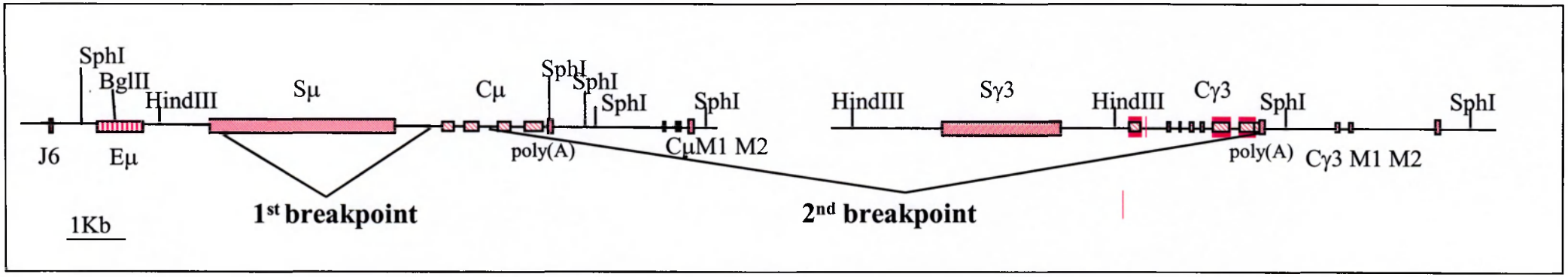


Figure 3.2

Southern blot analysis of switch μ/δ regions in MALT lymphoma **cases n°2 and n°1**. Genomic DNA was digested with the indicated restriction enzymes, electrophoresed, blotted and probed sequentially with various switch probes shown above each lane. Aberrant switch recombination fragments are indicated by the molecular weight and germline fragments by a "g". Sizes of the expected germline bands are reported in Fig.3.1 **a) Case n°2** shows two aberrant switch fragments identified by the 5'Sμ probe on a HindIII and SphI digest and two fragments identified by the 5'σμ probe on a BglII digest. **b) Case n°1** shows one aberrant switch fragment identified by the 5'Sμ probe on a HindIII digest and one aberrant fragment on a SphI digest which is identified also by the 3'Sμ probe. This case shows other two aberrant switch fragments with a 5'σμ probe on a BglI digest.



1st breakpoint sequence

HSJHCMU	CTGGGCTGAGCTGGGCTGAGCTGG	628
CASE #2	CTGGGCTGAGCTGGGCTGAGCTGGATTTTGTCCTGCGGGTCCTCAGGGAG	
HSIGCMUDE	TTTTGTCCTGCGGGTCCTCAGGGAG	124

2nd breakpoint sequence

HSIGCMUDE	GCGGCCAAGGGCAGGGGCTCGGGC	1018
CASE#2	GCGGCCAAGGGCAGGGGCTCGGGCCACGTGTACATACTTCCCGGGCACCCAGCA	
D78345	CGTGTACATACTTCCCGGGCACCCAGCA	10541

Figure 3.3

a) Graphic representation of the two breakpoints present in the 4.7Kb-5'Sμ aberrant fragment in **case n°2**. The two breakpoints were generated by a deletion from the Sμ region to just upstream of Cμ exon 1 and a second deletion starting from Cμ exon 3 to γ 3 sequences, upstream of the two membrane exons. Eμ=intronic enhancer, Sμ=switch μ region, Cμ=constant μ exons, M1,M2=exons for membrane form, Sγ=switch γ region, Cγ3=constant γ3 exons. **b)** Alignment of the breakpoint regions of case n°2 with germline GenBank sequences. Numbers indicate the nucleotide position at which the breakpoint occured within the corresponding GenBank sequence. These breakpoint sequences are available from GeneBank under accession numbers: AF156531, AF156532. HSJHCMU=GenBank nucleotide sequence of the IgH Sμ region, HSIGCMUDE=GenBank nucleotide sequence of the human IgH Cμ and Cδ genes, D78345=GenBank nucleotide sequence of the human Ig γ heavy chain.

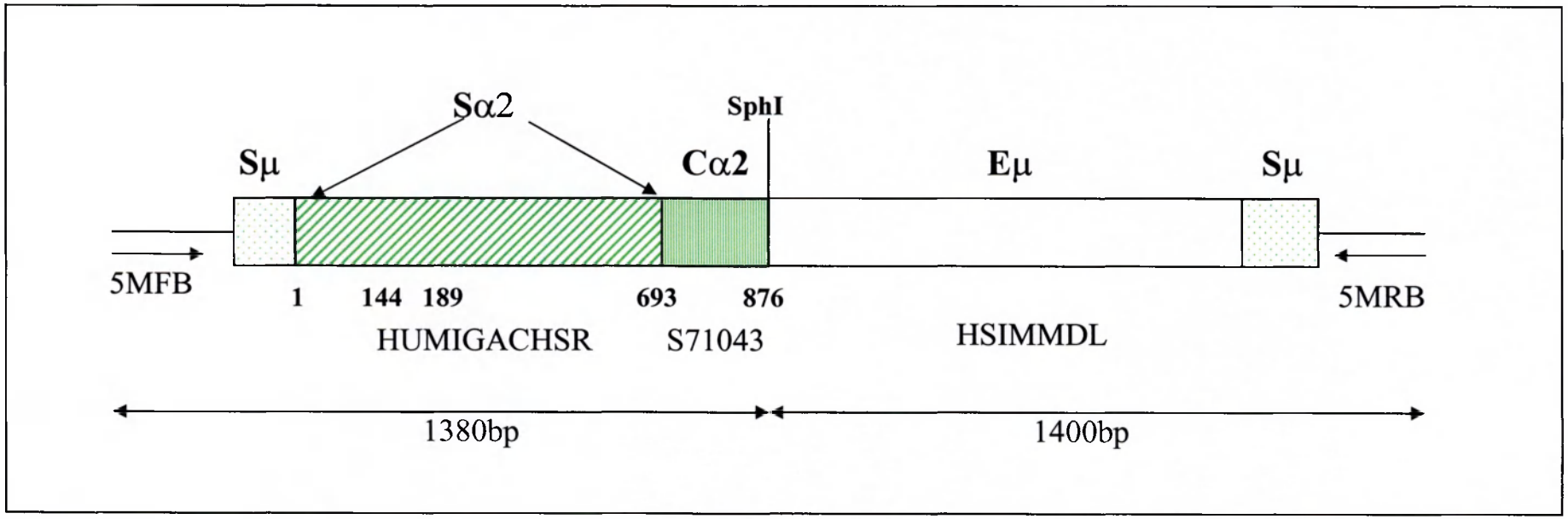


Figure 3.4

Graphic representation of the 2.8 Kb aberrant fragment obtained by long-distance inverse-PCR using the oligonucleotides 5MFA, 5MRA, 5MFB and 5MRB.

Sμ=switch mu region, Sα=switch alpha region, Cα2=constant alpha 2 region, Eμ=immunoglobulin heavy chain enhancer, HSIMMDL=human immunoglobulin D segment locus, HUMIGACHSR=human IgA switch alpha 1 region (Sα1), S71043=immunoglobulin A heavy chain allotype 2.

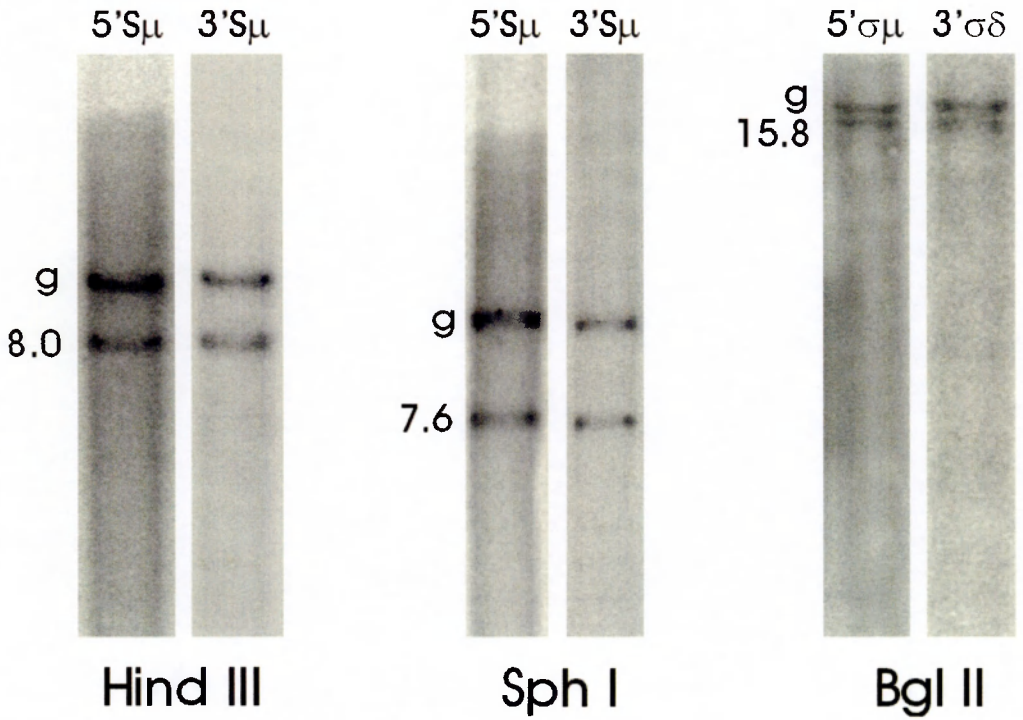


Figure 3.5

Southern blot analysis of switch μ/δ regions in **case n°3**. Probes for the μ and δ switch regions all cohybridized to germline fragments and to smaller rearranged fragments.

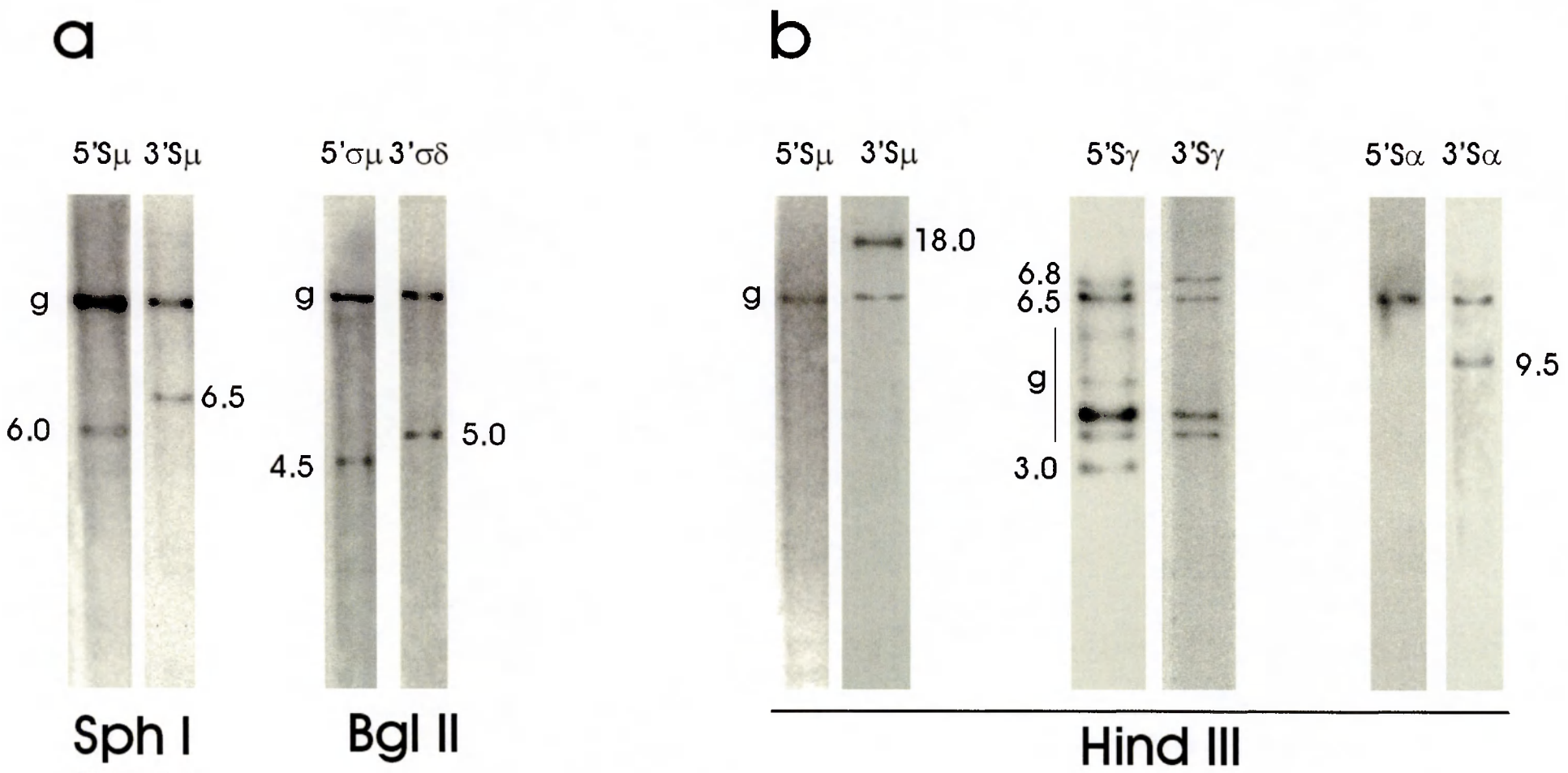


Figure 3.6

Southern blot analysis of switch regions in two patients with "high grade" MALT lymphoma. **a) Case n°6** shows two aberrant switch fragment identified by the 5'Sμ probe and by the 3'Sμ probe on a SphI digest. Two additional aberrant switch fragments are identified by the 5'σμ and the 3'σδ probes on a BglII digest. **b) Case n°7** presents one aberrant 3'Sμ fragment, three 5'Sγ fragments (two of them cohybridize with the 3γ probe) and one 3'Sα fragment, all on HindIII digest.

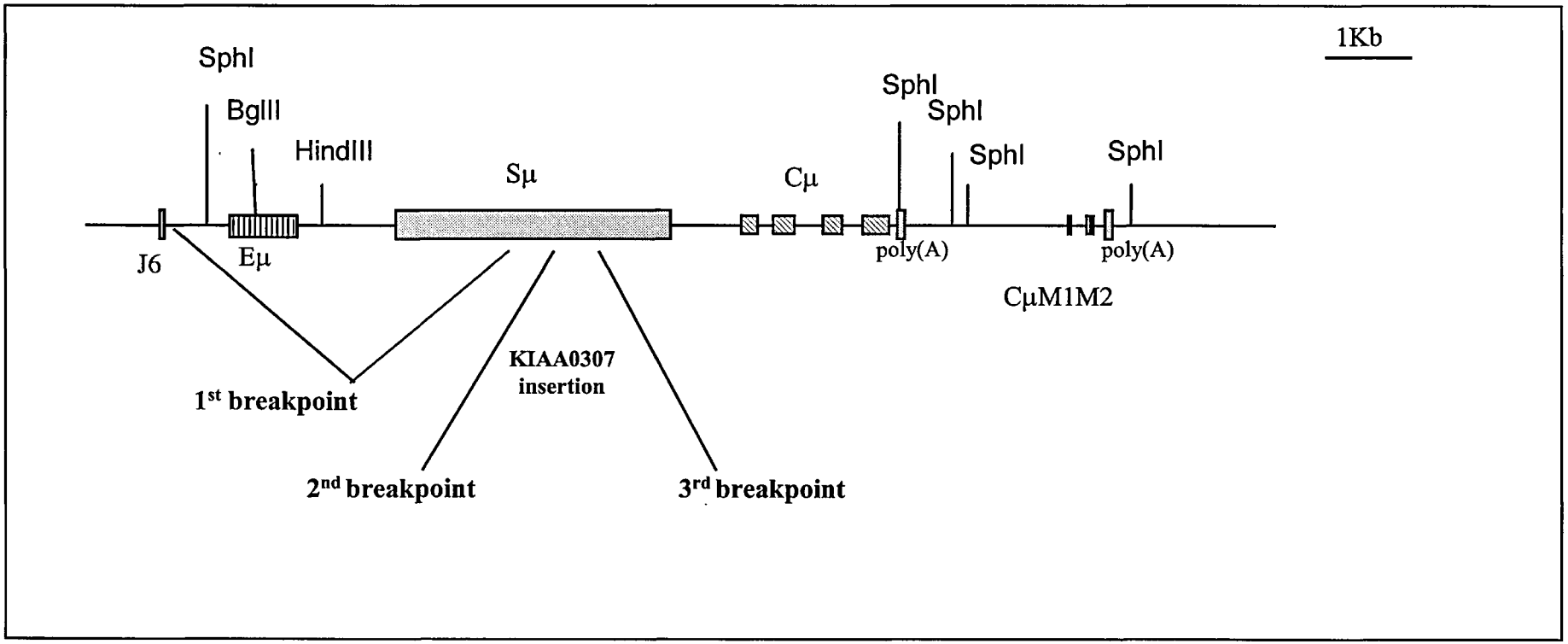


Figure 3.7 a

Graphic representation of the three breakpoints present in the aberrant 3'Sμ fragment in **case n°7**. The fragment was amplified by long distance inverse-PCR. Genomic DNA from case n°7 was digested with EcoRI, self-ligated and long distance inverse-PCR was performed using four primers (3MFA, 3MRA, 3MFB, 3MRB), internal to the 3'Sμ region. The three breakpoints are generated by a deletion from downstream JH6 to the middle of the Sμ region and by an insertion of a chromosome 15 gene (KIAA0307) into the Sμ region. Eμ=intronic enhancer, Sμ=switch μ region, Cμ=constant μ exons, M1,M2=exons for membrane form

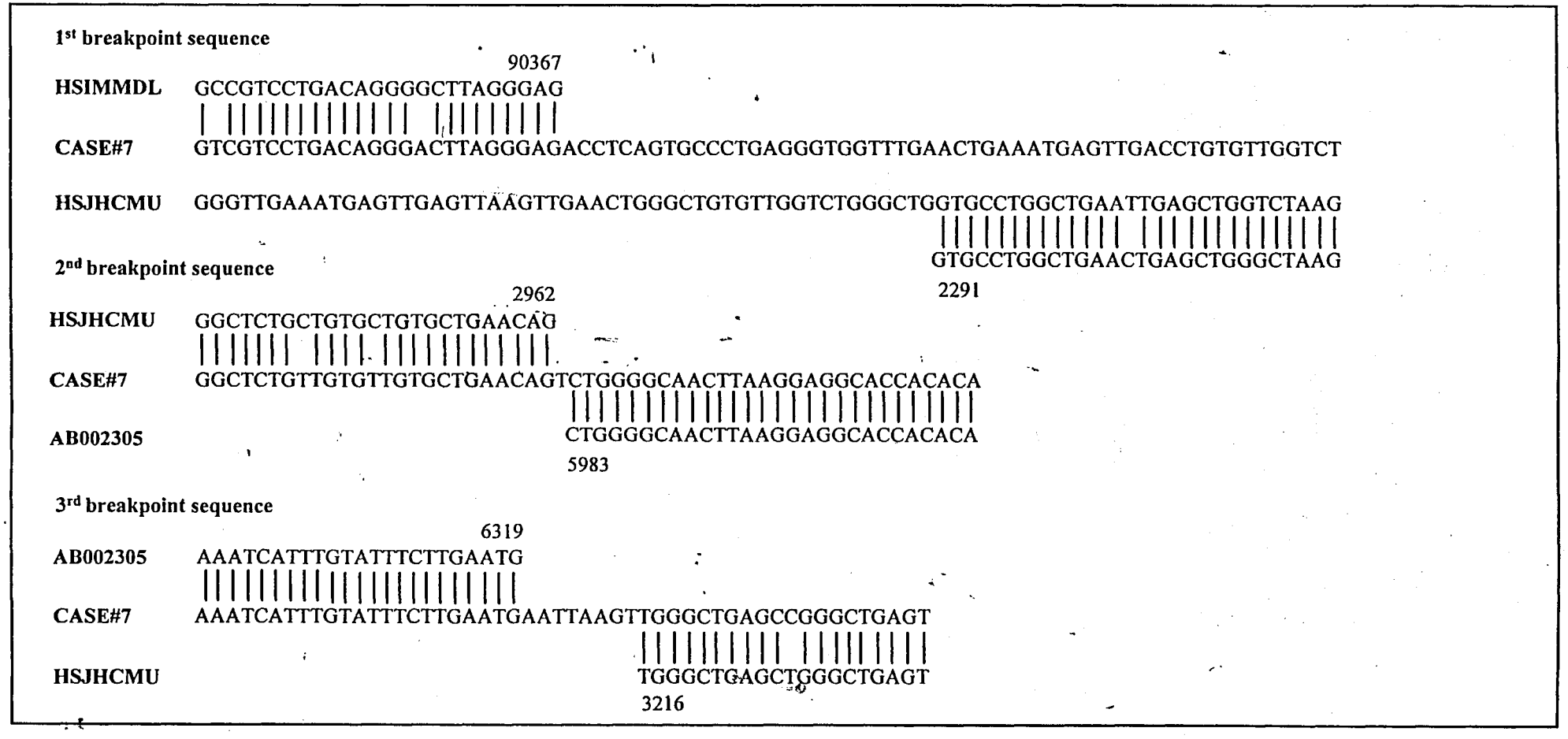


Figure 3.7 b

Alignment of the breakpoint regions of **case n°7** with germline GenBank sequences. Numbers indicate the nucleotide position at which the breakpoint occurred within the corresponding GenBank sequence. These sequences are available from GenBank under accession numbers: AF156533, AF156534, AF156535. HSIMMDL=GenBank DNA sequence of the human immunoglobulin D segment locus, HSJHCMU=GenBank nucleotide sequence of the IgH Sμ region, AB002305=GenBank nucleotide sequence of human mRNA for KIAA0307 gene.

Chapter 4

Analysis of variable heavy chain regions in gastric MALT lymphoma and correlation with isotype switch events

Variable heavy chain (V_H) analysis was performed on six cases of low-grade MALT lymphoma of the stomach. Two cases had been previously analyzed by Southern blotting for rearrangements in the IgH switch regions whereas four cases were retrieved specifically. The two cases previously analyzed (n° 1 and n° 2) are numbered 3 and 2 respectively in the following V_H analysis.

The four new cases, positive for IgM and negative for IgD, were first analyzed to determine the configuration of IgH switch regions. On different enzymatic digests (HindIII, SphI, BglII) one case (n° 1) showed evidence of aberrant isotype switch recombination in the Sμ region and three cases (n° 4, 5, 6) were in germline configuration. Representative hybridizations of the four new cases and of cases n°2 and n° 3 are shown in Fig. 4.1.

4.1 Variable heavy chain (V_H) genes amplification and analysis of common mutations

V_H genes were amplified in all 6 cases from genomic DNA using different primer pairs (see Materials and Methods) that amplify the complete V_H region from

framework 1 to the joining heavy region J_H (primers FR1 and JH) or the region from framework 2 to J_H (primers FR2 and JH).

Cases n° 1, 2, 3 and 4 were amplified from framework 1 to J_H and cases n° 5 and 6 from framework 2 to JH. In all but one case (n°4), a single monoclonal band was amplified (data not shown). In case n° 4 two bands were amplified, which were also indicative of monoclonality. Reaction products were then cloned and sequenced, and multiple clones for each case were screened. The V_H sequence of each clone was analyzed using the immunoglobulin Blast database and compared to the germline sequence with the highest homology to identify mutations. Based on the number of somatic mutations detected in the V_H genes, cases were classified as "unmutated" or "mutated".

Consistent with current convention, "***unmutated***" genes were defined as those with <2% differences from the most similar V_H germline, while "***mutated***" genes were those with >2% difference from the closest germline. Homology to published germline clones was searched at both the nucleotide and protein levels. A ***common mutation*** also called ***somatic mutation*** was defined as a nucleotide substitution shared among all clones of a given V_H.

Comparison of the V_H sequences with the known germline genes revealed that one case (n° 2) belonged to the V_H3 family, three cases (n° 1, 5, 6) to the V_H4 family and one case (n° 3) to the V_H5 family; case n°4 showed a biallelic V_HDJ_H rearrangement with V_H1 on one allele and V_H4 on the other allele (Table 4.1). A biallelic V_HDJ_H rearrangement (V_H1 and V_H4), was reported in other cases of low-grade and high-grade MALT lymphomas (Yumoto et al., 1999; Hallas et al., 1998; Hallas et al., 1998).

Frequencies of common mutations were determined on the ***dominant or consensus*** V_H sequence of each case. Somatic mutations were present in all V_H sequences and homology with the closest germline ranged from 89.5 to 98.8% (Table 4.1). The frequency of common mutations ranged from 1.2% to 10.5% (Table 4.2) and interestingly in patients n° 1, 2 and 3 was higher (mean 7.8%) than in patients n° 4, 5 and 6 (mean 2.4%) (p=0.05).

Each V_HDJ_H nucleotide sequence was then translated in order to check for amino acid replacements. V_H regions of all six low-grade MALT lymphomas revealed the correct frame with no stop codons. Expected replacement versus silent mutations (R/S) for CDRs and FRs were calculated according to the model of Chang and Casali (Chang and Casali, 1994). The ratios of replacement to silent (R/S) mutations in the complementarity-determining regions (CDRs) were higher than expected in four cases (n° 1, 2, 4, 5) and lower in the other two cases, whereas in FRs the ratios were lower than expected in all cases (Table 4.2). These features are characteristic of antigen-selected antibodies which are under positive pressure to provide the "best fit" for antigen while retaining framework structure to provide the scaffolding for the antigen-contacting CDRs (Chang and Casali, 1994). On the other hand, two cases (n° 3, 6) displayed lower R/S ratios in the CDRs than expected. A possible explanation for this feature may be selection by an autoantigen which usually provides negative selection pressure in CDRs to maintain autoantigen binding affinity (Bahler et al., 1992; Friedmann, 1991; Friedman et al., 1991). Consistently the CDR3 translated sequences in these 2 cases showed homology to autoantibodies.

4.2 Analysis of V_H region non-common mutations

Non-common (intraclonal) mutations were defined as nucleotide substitutions present in some, but not all clones of a given V_H .Only non-common mutations above the estimated Taq polymerase error-rate of approximately 0.2% were considered Based on this definition non-common mutations were present in two cases (n^o 5 and n^o 6). For each patient, at least three independent clones were analyzed. Table 4.3 gives the number of analyzed sequences for each patient and the frequency of non-common mutations, ranging from 0.2 to 3.5%. Fig. 4.2a shows the sequences of four independent clones in case n^o5 and Fig. 4.2b of three independent clones in case n^o6 with common and non-common mutations.

4.3 Third complementarity-determining region (CDR3) region analysis and homology to known antibodies

A stretch of seven consecutive nucleotide matches was considered indicative of a specific variable diversity (D) gene usage. In all but one case (n^o 1), the D segments showed significant homology to one of the known germline D genes (Table 4.1). One case showed usage of the D4 family, one case showed usage of both D5 and D4, two cases shared usage of the D3 family, while case n^o 4 showed D4 on one allele and D6 on the other allele. Cases 1 to 5 used J_H4 and case n^o 6 used J_H5.

For each patient, the consensus CDR3 nucleotide sequence was translated (Fig. 4.3). The length of the CDR3 in MALT lymphomas with aberrant isotype switch ranged from 9 to 13 amino acids (mean 11) and in cases without aberrant isotype switch ranged from 7 to 12 amino acids (mean 8.7). The length of CDR3 in all cases ranged from 7 to13 amino acids, in accord with reports (2 to 20 amino acids) by others in

gastric low-grade MALT lymphomas (Hallas et al., 1998; Bertoni et al., 1997; Thiede et al., 1998; Qin et al., 1995; Du et al., 1996). Surprisingly two cases (4 and 5) showed similar CDR3 sequences. This similarity suggests the presence of a common selecting antigen, as previously described in gastric MALT lymphomas by (Bertoni et al., 1997). Although the two cases have similar CDR3 they differ in the type of V(D)J rearrangement and in the level of intraclonal diversity. Case 4 shows a biallelic V(D)J rearragement, generating V_H1 on one allele and V_H4 on the other allele while case 5 shows a single V_H4 allele. Moreover case 4 shows no non-common mutations unlike case 5 that shows a high load of intraclonal diversity (0 and 23 in Table 4.3). Two N segments were added in the three cases with aberrant isotype switch while only one N segment was added in the other three cases.

Comparison of CDR3 amino acid sequences with GenBank databases revealed high homology to known antibodies in four cases. Case n° 3 showed 75% identity with an autoantibody from a patient with rheumatoid arthritis and case n° 6, 72% identity with an autoantibody from a patient with lupus. Cases n° 4 and n° 5 showed identity with an antibody isolated from a patient with Kawasaky disease (77% and 83%, respectively), while CDR3 from cases n° 1 and n° 2 showed no homology to any known antibody.

Table 4.1. Use of V_H, D, J_H gene segments by six gastric low-grade MALT lymphomas

Case	V_H Family	Closest Germline	(%) Homology	D Gene	J_H Gene
1	V_H4	V_H4-59	95.9	NA[b]	J_H4
2	V_H3	V_H3-7	89.5	D3-16	J_H4
3	V_H5	V_H5-51	91.2	D5-12/D4-17	J_H4
4	V_H1	V_H1-2	100	D6-19	J_H4
	V_H4	V_H4-59	97.6	D4-17	J_H4
5[a]	V_H4	V_H4-59	96.3	D4-17	J_H4
6[a]	V_H4	V_H4-59	98.8	D3-10	J_H5

[a]these cases were amplified from FR2 to J_H

[b]not assigned

Table 4.2

Distribution of common mutations in the six V_H genes

Case	Common Mutations (%)	CDRs				FRs			
		R	S	R/S	Expected R/S	R	S	R/S	Expected R/S
1	12 (4.1)	4	0	∞	4.55	4	4	1	2.63
2	31 (10.5)	6	1	6	4.08	14	10	1.4	3.15
3	26 (8.8)	6	2	3	3.83	12	6	2	2.95
4[a]	7 (2.4)	2	0	∞	4.55	3	2	1.5	2.63
5[b]	7 (3.7)	2	0	∞	4.55	3	2	1.5	2.63
6[b]	2 (1.2)	0	0	0	4.55	1	1	1	2.63

[a]analysis of the V_H4 allele

[b]analysis of the FR2-J_H region

Common mutations were defined as a nucleotide substitution shared among all clones of a given V_H; colums headed with R and S indicate the number of deduced replacement (R) or silent (S) mutations from the proposed germline V_H nucleotide sequence. Expected R/S refers to the ratio of all possible R to S mutations as described by Chang and Casali, 1994.

Table 4.3

Aberrant isotype switch, non-common mutations, number of analyzed sequences and frequency of non-common mutations in 6 cases of gastric low-grade MALT lymphoma

Case	Aberrant switch	Non-common mutations	Sequences analyzed (n°)	Frequency (%)
1	Yes	0	5	0
2	Yes	0	4	0
3	Yes	2	3	0.2
4[b]	No	0	4	0
5[a]	No	23	4	3
6[a]	No	18	3	3.5

[a] analysis of the FR2-J_H region

[b] analysis of the V_H4 allele

Non-common (intraclonal) mutations were defined as a nucleotide substitution present in some, but not all clones of a given V_H.

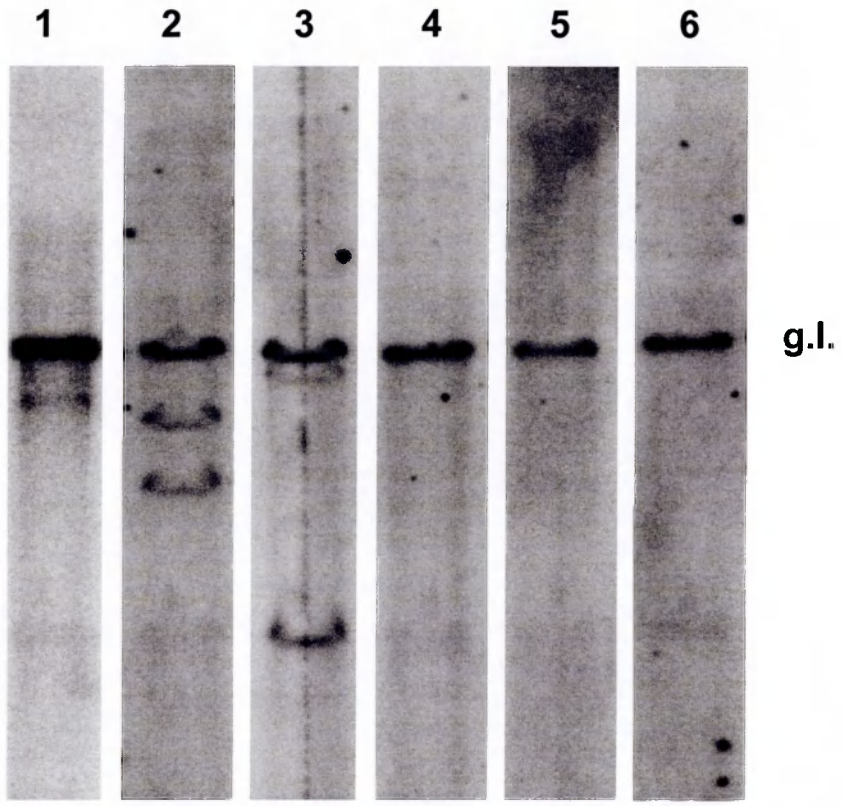


Figure 4.1

Southern blot analysis of IgH switch regions in six gastric low-grade MALT lymphomas. Genomic DNA, digested with SphI (cases n° 1 and n° 4) or HindIII (cases n° 2, 3, 5, 6), was electrophoresed, blotted and probed sequentially with the 5' switch mu (5'Sμ) probe. Aberrant isotype switch recombination fragments are indicated by an arrow and germline fragments by a "g. l.".

Case 5.

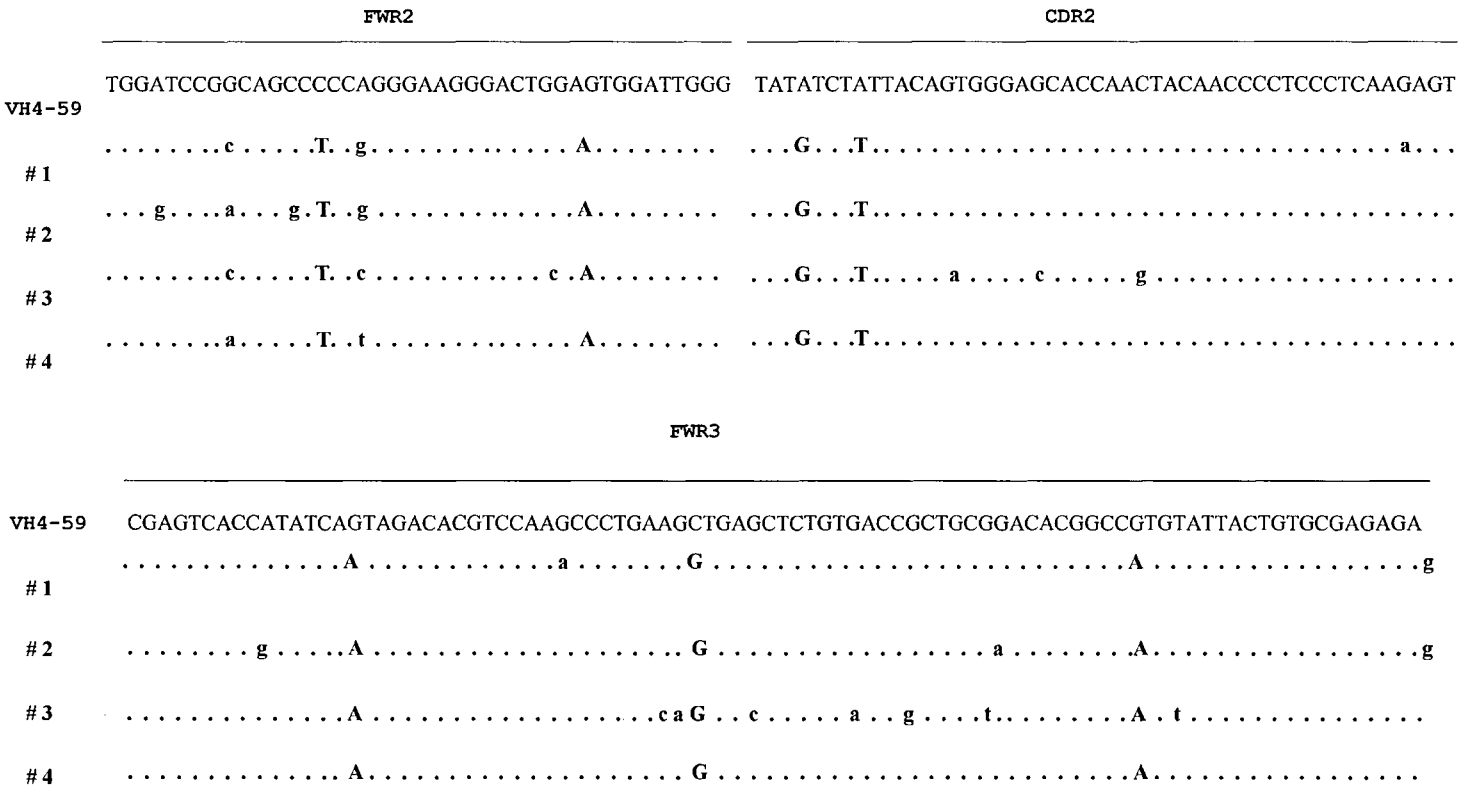


Figure 4.2a

V_H gene intraclonal heterogeneity in gastric low-grade MALT lymphoma case 5. Non-common mutations are indicated in lower case and common mutations in upper case.

Case 6.

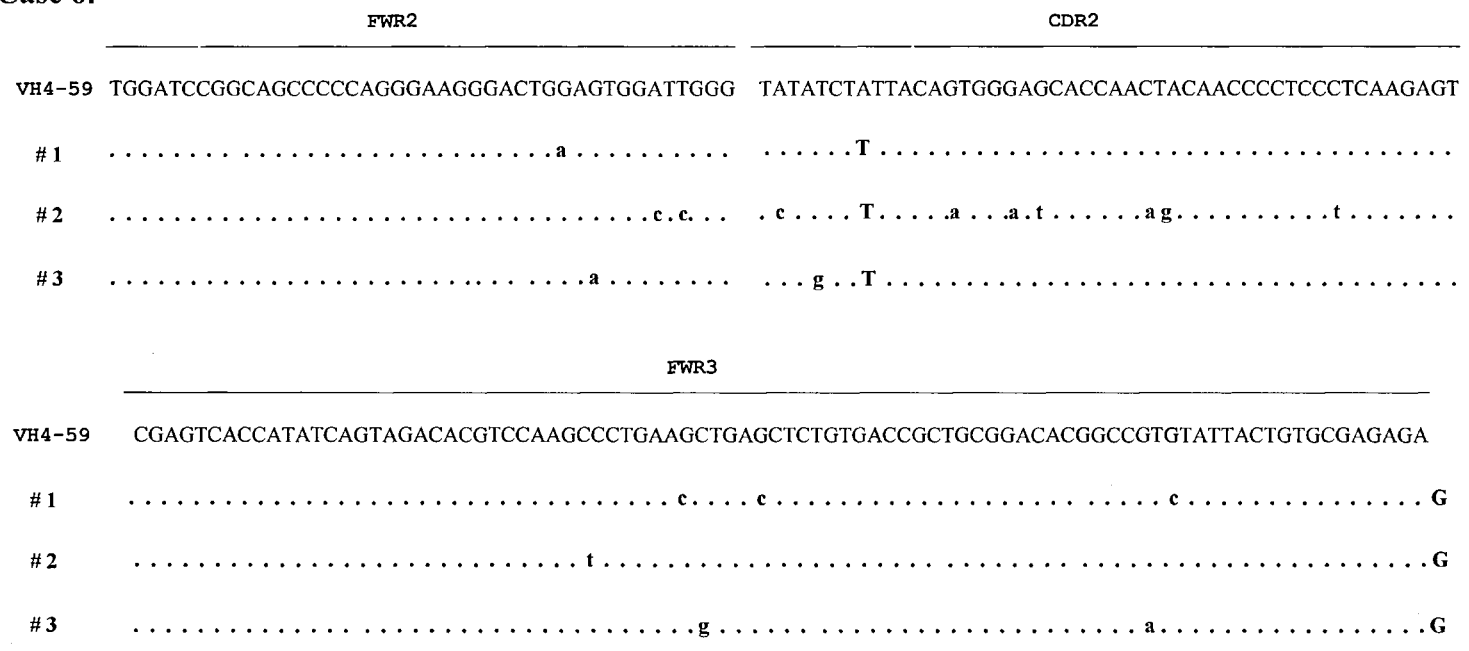


Figure 4.2b

V_H gene intraclonal heterogeneity gastric low-grade MALT lymphoma case 6. Non-common mutations are indicated in lower case and common mutations in upper case.

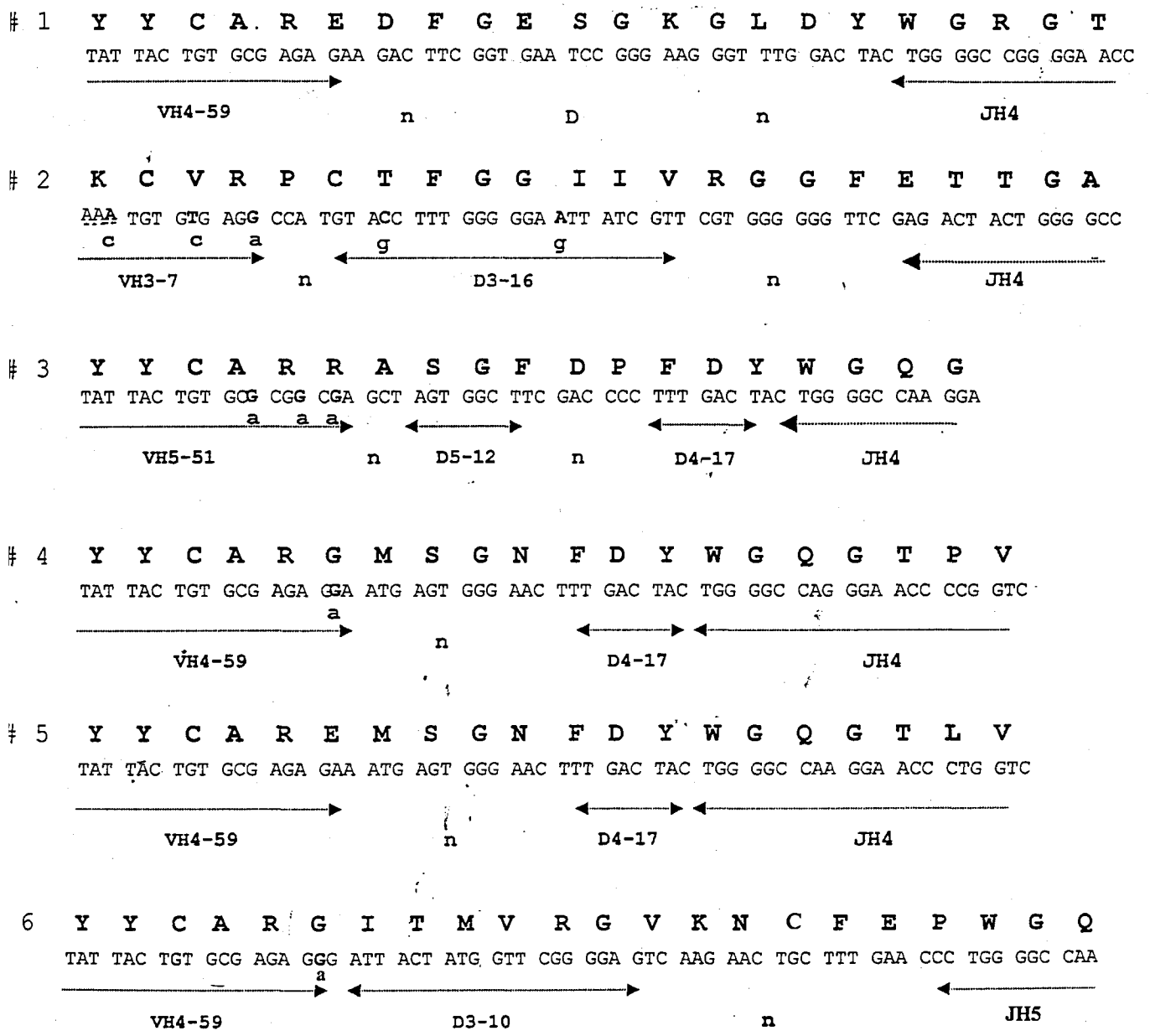


Figure 4.3

CDR3 consensus sequences of gastric low-grade MALT lymphomas. V_H PCR products were cloned and multiple clones sequenced. The deduced amino acid sequence of the consensus clone and the V_HDJ_H boudaries are shown. Nucleotide substitutions were identified by comparison of the consensus sequence with the published germline family sequence.

Chapter 5

Analysis of the immunoglobulin switch regions and of the variable heavy chain genes in B-cell Chronic Lymphocytic Leukemia

5.1 B-cell Chronic Lymphocytic Leukemia (B-CLL) patients and phenotypic characterization

Forty patients with clinical features of B-CLL were randomly chosen among a series of 145 cases. The mean age of patients at diagnosis was 61.5 years (range, 49 to 83). Peripheral blood of these patients revealed expansion of cells with the typical phenotype of B-CLL. $CD5^+/CD19^+$ neoplastic cells represented from 60 to 98% of peripheral mononuclear cells and were $CD23^+$ and $CD22^-$, with low or absent CD79b expression. Surface immunoglobulin expression was evaluated with FITC-labelled F(ab')2 rabbit polyclonal antibodies against human IgM, IgD, IgG, Igk and Igλ chains (DAKO).

5.2 Southern blot and PCR analysis of Sμ regions in B-CLL cases.

Forty B-CLL patients were initially screened for immunoglobulin isotype; 28 had IgM^+ B leukemic cells, 8 had IgM^+/IgD^+, two IgG^+, one IgD^+ and one had non-producing cells (Table 5.1). DNA was extracted from peripheral blood lymphocytes of the 38 patients with "unswitched" isotype, i.e., no evidence of productive isotype

switch, and analyzed by Southern blotting using the two probes localized upstream (5'Sμ) and downstream (3'Sμ) of the Sμ region (Fig. 2.1A).

On SphI digestion, DNA from 25 of the 38 patients showed a Sμ region in germline configuration, while the remaining 13 patients showed additional rearranged bands (Fig. 5.1). Hybridization with the 5'Sμ probe, revealed seven cases (cases n° 1,2,5,7,27,36,40) with one rearranged band and six cases (cases n° 3,4,18,20,31,35) with two rearranged bands. With the 3'Sμ probe, ten cases (cases n° 1,2,3,4,5,7,18,20,27,40) presented one rearranged band and three cases presented two rearranged bands (cases n° 31,35,36). Interestingly, in all but two of these cases (35,36), the rearranged fragments identified by the 5' and 3'Sμ probes cohybridized and were about 0.5-4 kb smaller than the germline fragment. The rearranged bands all had different sizes (Table 5.2), excluding the possibility that they were polymorphic alleles. The cohybridization of the two Sμ probes suggested an internal deletion in the Sμ region (Mukherjee et al., 1993; Zhang et al., 1995).

Indeed PCR using primers 5MFA/3MRA or additional primer pairs located upstream and downstream to 5MFA/3MRA indicated the presence of deletions in the Sμ regions. In all 13 cases with Sμ rearrangements, a band of reduced size compared to the germline was amplified (Fig. 5.2). Cases in Figure 5.2A were amplified with primers 5MFA/3MRA, which amplify a fragment of 3.9 kb when the Sμ region is in germline configuration, whereas cases in Figure 5.2B were amplified with primers 5'σμF/3MRB, which amplify a 5.1 kb fragment when the Sμ region is in germline configuration. In three cases (cases n° 20,35,40), PCR amplification produced very faint bands which are not shown. In all cases, the reduction in size of the PCR-amplified products corresponded to the reduction observed for the rearranged bands

on the Southern blot (Table 5.2).

Specifically, genomic DNA of ***patients 1, 2, 5, 7, 27, and 40*** presented the same hybridization pattern with the 5' and 3' Sμ probes on a SphI digest, and showed the germline bands (9 kb) and additional smaller rearranged bands (Fig. 5.1). PCR amplification with primers 5MFA/3MRA produced bands of reduced size compared to the germline (Fig. 5.2A and Table 5.2). Thus, in all these cases, a deletion internal to the Sμ region occurred in one allele.

Patients 18 and 20 showed the germline bands, two rearranged bands with the 5'Sμ probe and one rearranged band with the 3'Sμ probe (Fig. 5.1). PCR amplification of the Sμ region produced bands corresponding to the allele recognized by both 5' and 3'Sμ probes (Fig. 5.2B and Table5.2).

In ***Case 3***, Sμ deletions on both alleles were observed. Hybridization with the 5'Sμ probe revealed the germline band and two rearranged bands of 7.5 kb and 7 kb, while the 3'Sμ probe identified the germline band and only the 7 kb band (Fig. 5.1). PCR using primers 5MFA/3MRA amplified a 1.8 kb band (Fig. 5.2A) corresponding to the 7 kb allele. On this allele, a 2 kb deletion occurred. PCR using primers 5MFB and CmR1, that amplify a 4.1 kb fragment when the Sμ region is in germline configuration, amplified two bands of 2.3 kb and 2.5 kb, indicating that the 7.5 kb allele has a 1.6 kb deletion towards the 3' end and, thus, could not be amplified with primer 3MRA.

Even ***Case 31*** showed Sμ deletions on both alleles. Indeed, the two Sμ probes

hybridized to two rearranged fragments of 6.4 kb and 6 kb but failed to hybridize to the germline band (Fig. 5.1). PCR with primers 5MFA/3MRA amplified the Sμ region in only one of the two rearranged alleles, producing one band of 1 kb (Fig. 5.2A). To amplify the Sμ region of both alleles, additional primer pairs were used. 5MFB and 3MRB produced two bands of 0.8 kb and 0.4 kb, indicating a 2.8 kb deletion between 5MFB and 3MRB on the 6.4 kb allele, and a 3.2 kb deletion on the 6 kb allele.

In ***Case 36***, hybridization with the 5'Sμ probe revealed a band of larger size than the germline, while hybridization with the 3'Sμ probe showed the same large band and a rearranged band of 5.5 kb (Fig. 5.1). PCR with primers 5MFA/3MRA gave no amplified products, while primers 5'σμF/3MRB amplified a 1.9 kb band (Fig. 5.2B) indicating a 3.2 kb deletion towards the 5' end of one allele.

Similarly, ***Case 35*** presented bands larger than the germline with the two Sμ probes. These larger bands might contain a restriction site polymorphism or may be generated by insertions (Shapira et al., 1991), or by inversions (Laffan and Luzzatto, 1992).

Sequencing of the PCR-amplified fragment from ***Case 4*** confirmed the presence of an internal Sμ deletion. This case showed the germline bands and two rearranged bands of 8 kb and 5.2 kb with the 5'Sμ probe and one rearranged band of 5.2 kb with the 3'Sμ probe (Fig. 5.1). No reduced size bands were amplified with primers 5MFA/3MRA, whereas primers 5'σμF/3MRB amplified a 1.25 kb band (Fig. 5.2B). The sequence of the 1.25 kb band (Fig. 5.3) showed a germline configuration from

the 5'σμF position in HSIMMDL (90885) to position 92021. After the breakpoint (ending at position 3671 in HSJHCMU), the sequence continued until position 3803 in HSJHCMU (3MRB position). Thus, a 3.75 kb deletion occurred on this allele between position 92021 in HSIMMDL and position 3671 in HSJHCMU. Additional PCRs did not amplify any product from the other allele, which might be involved in a translocation, or any other chromosomal rearrangement.

5.3 Localization of Sμ deletions in the V_HDJ_H rearranged allele.

To define the allelic localization of Sμ deletions, cases bearing these deletions were amplified by PCR using a "touchdown" strategy and primers in the V_H region and in the 3' part of the Sμ region (see Materials and Methods). Sizes of the V_HDJ_H rearranged fragments varied depending on the presence or absence of the Sμ deletion. Using long-distance PCR conditions, it was possible to amplify the allele with the V_HDJ_H and Sμ deletion in 8 out of 13 cases (Fig. 5.4). In only one case (n^o 7) the Sμ deletion occurred on the unrearranged allele. Case n^o 12, that presents a V_HDJ_H rearranged allele without Sμ rearrangements, was used as control. It was not possible to amplify DNA from the remaining 4 cases by long-distance PCR.

5.4 Analysis of variable heavy chain genes in B-CLL and correlation with isotype switch events

Genomic DNA of the 38 B-CLL patients analyzed for rearrangements in the IgH switch regions was also analyzed for the presence of somatic mutations in the V_H regions. Amplification of the V_H region using consensus primers in the framework 1

region (FR1c) and in the joining heavy chain region (JHc) resulted in a PCR fragment of about 350 bp.

Sixteen cases expressed V_H1 family genes, 14 cases V_H3, five cases V_H4, two cases V_H2, and onr case expressed both V_H1 and V_H3 (Table 5.3). The number of somatic mutations was determined by comparing the V_H sequences to the germline genes with the highest homology using the Ig Blast database. As previously reported "***unmutated***" genes were defined as those with <2% differences from the most similar V_H germline, while "***mutated***" genes were those with >2% difference from the closest germline. Somatic mutations ranging from 2.3% to 11.6% were observed in 16 cases while the leukemic cells of 21 patients had V_H genes with ≥98% sequence homology with the nearest germline gene. In the remaining case presenting a productive rearrangement of both variable regions, a V_H1-2 in germline configuration and a V_H3-48 in mutated form was observed. A biallelic V_HDJ_H rearrangement was reported in other cases of B-cell CLL (Rassenti and Kipps, 1997) and it is not an infrequent event, since about 5% of B-CLL cells lack immunoglobulin heavy chain allelic exclusion. Of the two productive V_H genes, one was mutated and the other was not, consisistent with the finding that B-CLL without allelic exclusion can express one allele without somatic mutation along with the other allele that has undergone somatic mutation (Rassenti and Kipps, 1997). Cases with V_H mutations showed overusage of the V_H3 family while cases without V_H mutations showed over usage of the V_H1 family. In 8/17 cases with V_H mutations, R/S ratios in the complementary determining regions were higher than expected from random distribution while in the framework regions the ratios were lower.

No correlation between the presence of Sμ rearranged bands and V_H gene mutations

was observed, since 4 of 13 cases (32%) with a deletion in the Sμ region showed V_H mutations, and 13 of 25 of cases (52%) without deletion in the Sμ showed V_H mutations (p=0,13).

5.5 Clinical observations

The clinical course of 22 patients with a follow-up period of at least five years was related to the presence of Sμ rearranged bands and V_H mutations. The presence of switch rearrangements alone was not indicative of the clinical course. Indeed, out of eight cases with switch rearrangements five (cases n° 1,20,27,35,36) underwent disease progression (62.5%) whereas out of 14 cases without switch rearrangements six (no13,19,21,24,26,30) underwent progression (42,8%) (p=0,24). On the contrary, the presence of V_H mutations was indicative of the clinical course. Out of 11 cases with V_H mutations only three cases (cases n° 13,24,35) were in progression (27,2%) whereas out of 11 cases without V_H mutations eight cases (cases n° 1,19,20,21,26,27,30,36) (72%) were in progression (p=0,04).

Table 5.1. Immunoglobulin isotype and presence of Sμ rearrangements in B-CLL patients

Patients	Immunoglobulin isotype	Sμ rearranged bands
1	μ/δ	yes
2	μ	yes
3	μ	yes
4	μ	yes
5	μ	yes
6	μ/δ	no
7	μ	yes
8	μ	no
9	μ	no
10	μ	no
11	μ	no
12	μ	no
13	μ	no
14	μ	no
15	μ	no
16	μ	no
17	γ1	not assayed
18	μ	yes
19	μ	no
20	μ	yes
21	μ	no
22	μ	no
23	μ	no
24	μ/δ	no
25	μ/δ	no
26	μ/δ	no
27	μ	yes
28	μ	no
29	γ	not assayed
30	μ	no
31	μ	yes
32	μ	no
33	μ	no
34	μ/δ	no
35	non-producing	yes
36	μ/δ	yes
37	μ	no
38	δ	no
39	μ	no
40	μ/δ	yes

Table 5.2. Southern blot and PCR results for the 13 cases with Sμ rearrangements.

Case	Rearranged fragments size (kb)[a]		PCR fragments size (kb)	Primer pairs	PCR deletion size (kb)
	5'Sμ	3'Sμ			
1	8	8	3	5MFA-3MRA	0.9
2	8.5	8.5	3.5	5MFA-3MRA	0.4
3	7	7	1.8	5MFA-3MRA	2.1
	7.5		2.5	5MFB-CmR1	1.6
4	5.2	5.2	1.25	5'σμF-3MRB	3.75
	8				
5	7.5	7.5	2.5	5MFA-3MRA	1.4
7	7.2	7.2	2.3	5MFA-3MRA	1.6
18	6	6	2.5	5'σμF-3MRB	2.6
	8				
20	8.5	8.5	3.5	5MFA-3MRA	0.4
	6.5				
27	6.4	6.4	1.5	5MFA-3MRA	2.5
31	6.4	6.4	1 and 0.8	5MFA-3MRA and 5MFB-3MRB	2.8
	6	6	0.4	5MFB-3MRB	3.2
35	6.1	6.1	0.9	5MFA-3MRA	2.9
36		5.5	1.9	5'σμF-3MRB	3.2
40	8.5	8.5	3.5	5MFA-3MRA	0.5

[a]only rearranged fragments smaller than the germline are indicated

Table 5.3. V_H somatic mutation analysis and presence of Sμ rearrangements in B-CLL patients

P	Sμ rearranged bands	Germline VH	Homology %	FRs R/S o	FRs R/S e	FRs p	CDRs R/S o	CDRs R/S e	CDRs p
1	yes	VH1-2	99.7	0	nd	nd	0	nd	nd
2	yes	VH3-15	96.7	0.5	2.83	0.019[a]	3	4.06	0.190
3	yes	VH3-30.5	100	0	nd	nd	0	nd	nd
4	yes	VH1-69	100	0	nd	nd	0	nd	nd
5	yes	VH1-2	100	0	nd	nd	0	nd	nd
6	no	VH1-2	100	0	nd	nd	0	nd	nd
7	yes	VH3-72	97.7	4	2.80	0.293	>2	4.06	0.265
8	no	VH1-69	99	0	nd	nd	2	3.78	0.078
9	no	VH3-7	91.2	3.3	2.85	0.024[a]	5.5	4.08	0.002[a]
10	no	VH1-69	100	0	nd	nd	0	nd	nd
11	no	VH3-21	97.6	2	2.82	0.096	>4	4.03	0.019[a]
12	no	VH4-59	94.5	0.62	2.64	0.025[a]	0.5	4.55	0.153
13	no	VH2-70	96	2	2.87	0.198	2	4.09	0.292
14	no	VH2-70	100	0	nd	nd	0	nd	nd
15	no	VH3-11	100	0	nd	nd	0	nd	nd
16	no	VH1-2 VH3-48	100 95.6	0 1	nd 2.82	nd 0.035[a]	0 >5	nd 4.04	nd 0.049[a]
18	yes	VH1-2	92.6	1.5	2.98	0.045[a]	6	3.94	0.102
19	no	VH4-4	100	0	nd	nd	0	nd	nd
20	yes	VH1-69	100	0	nd	nd	0	nd	nd
21	no	VH4-39	100	0	nd	nd	0	nd	nd
22	no	VH3-74	91.9	1.25	2.84	$2.34x10^{-4}$[a]	6.5	4.05	$5.50x10^{-5}$[a]
23	no	VH3-48	96.6	0.25	2.82	0.003[a]	4	4.04	0.066
24	no	VH3-7	93.6	>6	2.84	0.014[a]	12	4.05	$1.40x10^{-5}$[a]
25	no	VH1-69	99	0.5	3.01	0.305	0	nd	nd
26	no	VH1-2	100	0	nd	nd	0	nd	nd
27	yes	VH3-30	98.6	0	nd	nd	1	4.01	0.129
28	no	VH1-2	99.3	>2	2.97	0.141	0	nd	nd
30	no	VH1-69	99.3	1	3.01	0.487	0	nd	nd
31	yes	VH1-46	100	0	nd	nd	0	nd	nd
32	no	VH1-69	100	0	nd	nd	0	nd	nd
33	no	VH1-8	97	2	2.95	0.027[a]	5	3.90	0.010[a]
34	no	VH3-53	88.4	0.57	2.83	$3.61x10^{-5}$[a]	5	4.08	0.032[a]
35	yes	VH4-34	95.2	0.28	2.65	0.001[a]	4	4.44	0.138
36	yes	VH1-3	98.6	1	2.99	0.170	>2	3.94	0.128
37	no	VH3-48	89.2	1.5	2.82	0.011[a]	11	4.04	0.012[a]
38	no	VH4-34	92.5	1.5	2.64	0.055	2.5	4.47	0.165
39	no	VH3-33	94.3	1.2	2.85	0.037[a]	>6	4.01	0.046[a]
40	yes	VH3-30	98.7	0	nd	nd	1	4.01	0.129

P= patients; **CDRs** = Complementarity-determining regions (CDR1 and CDR2); **FRs** =. Framework regions (FR1, FR2 and FR3); **R** = number of replacement mutations; **S** = number of silent mutations; **R/S o**= observed; **R/S e (expected)** = mutation ratio of the total possible R to total possible S mutation; **P** = probability; [a]= statistically significant (P<.05)

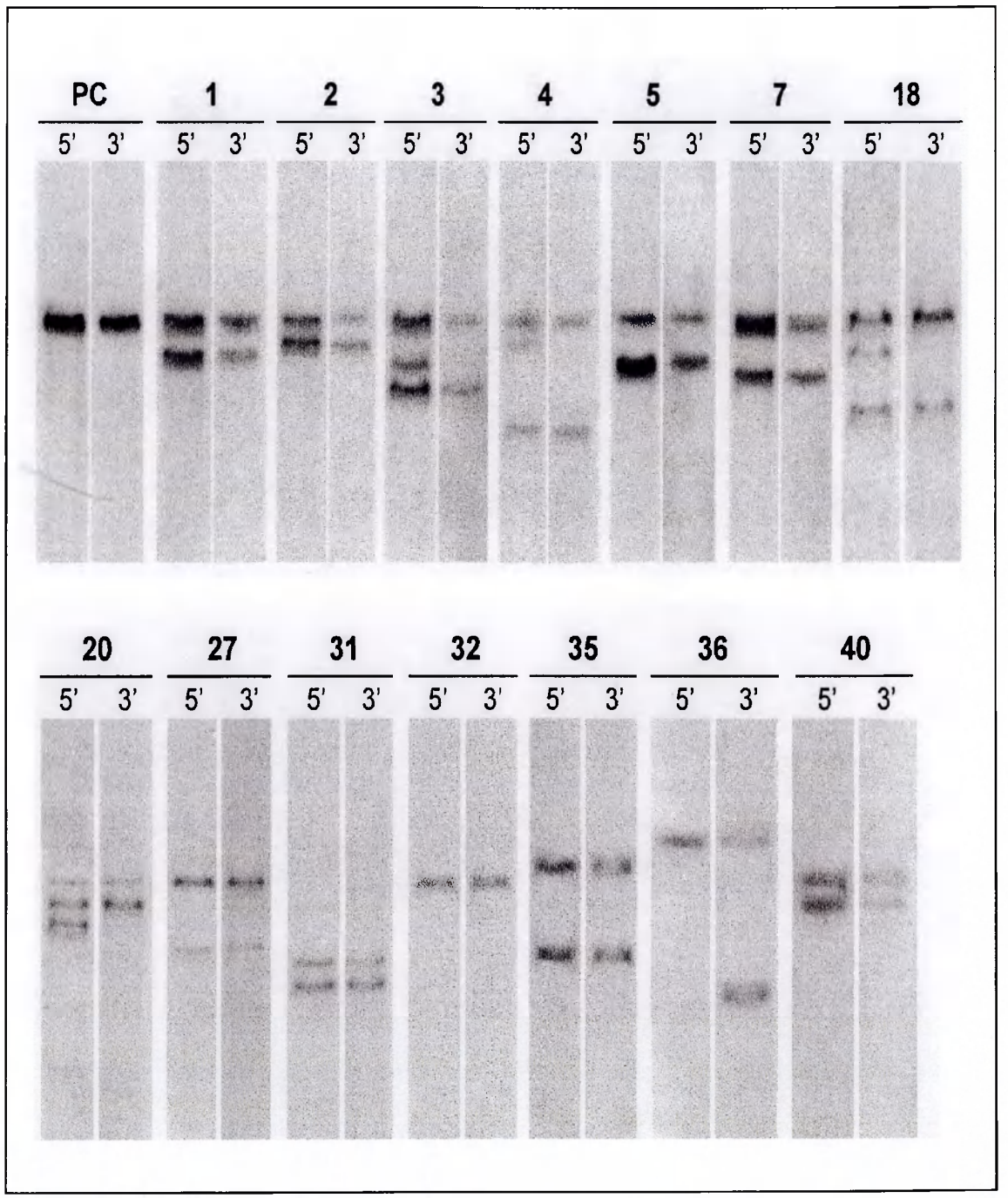


Figure 5.1

Southern blot analysis of Sμ regions in B-CLL patients. Genomic DNA of B-CLL patients was digested with SphI restriction enzyme, electrophoresed, blotted and probed sequentially with 5'Sμ and 3'Sμ probes. The hybridization pattern of 13 cases with rearranged Sμ bands and one case (n° 32) in germline configuration is shown.
PC=placental genomic DNA used as germline control.

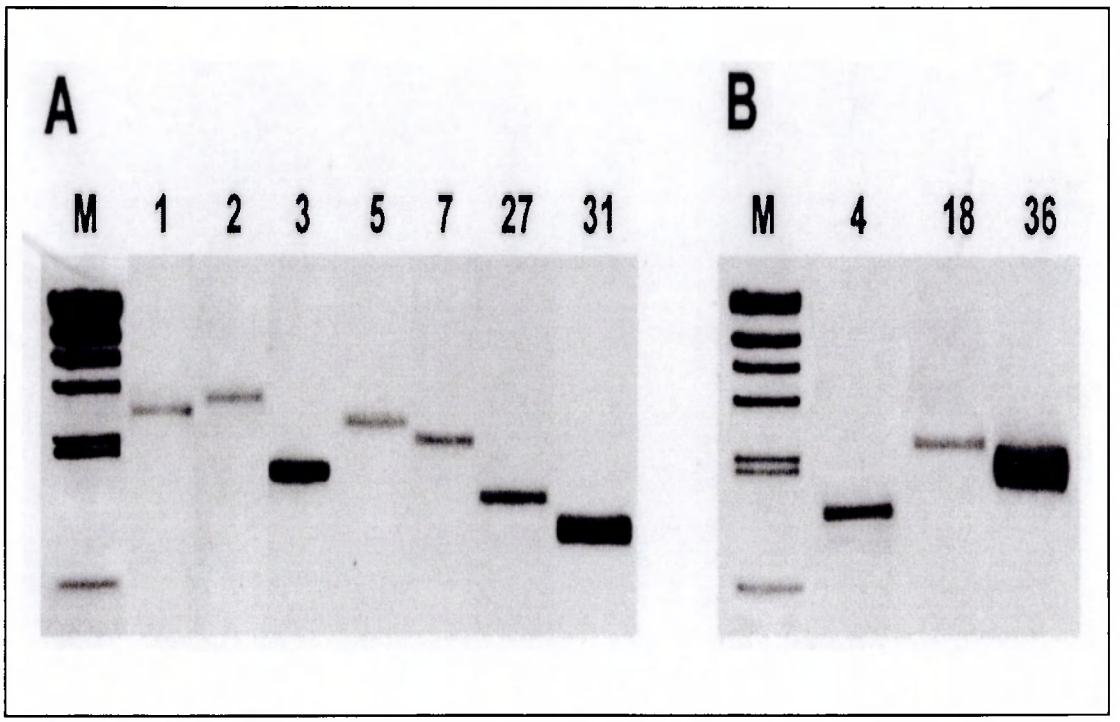


Figure 5.2

PCR amplification of the Sμ region in patients with Sμ rearranged bands. A. Amplification of seven B-CLL cases with 5MFA/3MRA primer pair. **B.** Amplification of three cases with 5'σμF/3MRB primer pair.

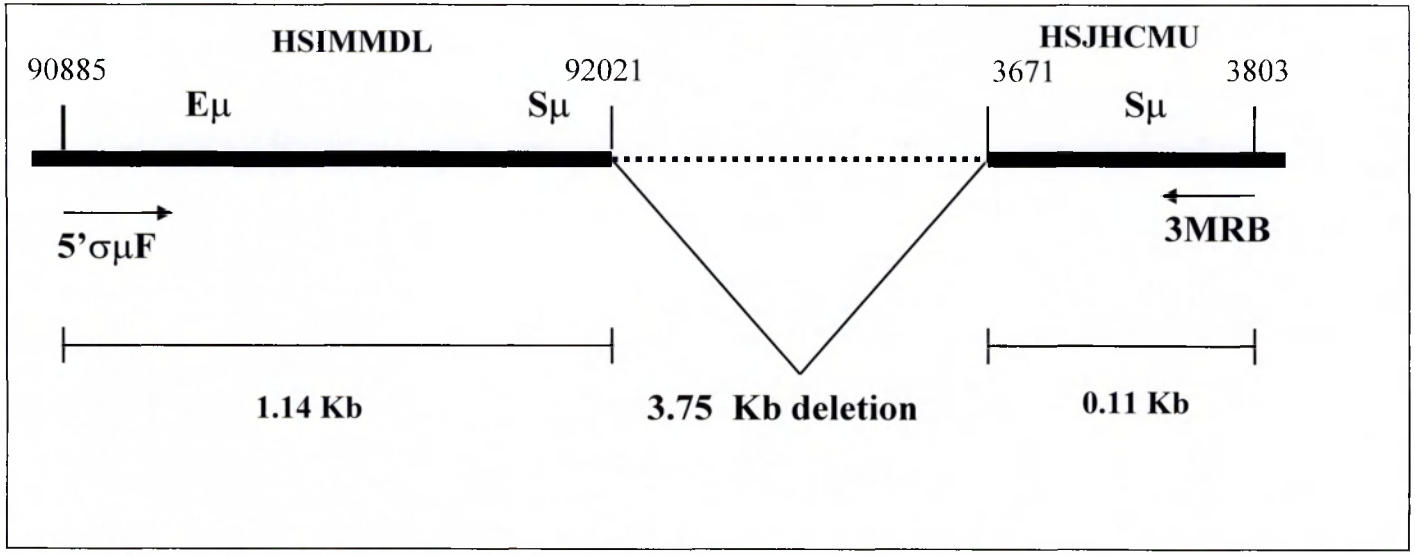


Figure 5.3

Graphic representation of the breakpoint present in the rearranged Sμ fragment of B-CLL case 4. The fragment was PCR-amplified using the 5'σμF/3MRB primer pair. Eμ=IgH enhancer; Sμ=switch mu region, HSIMMDL=GenBank DNA sequence of the human immunoglobulin D segment locus, HSJHCMU=GenBank nucleotide sequence of the IgH Sμ region

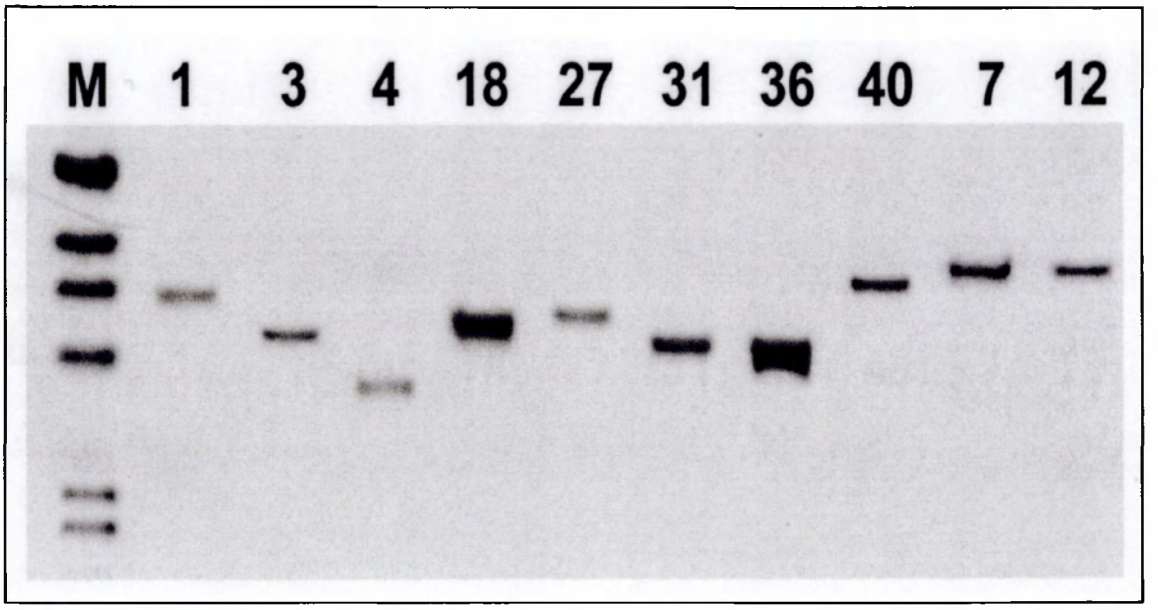


Figure 5.4

Amplification of the rearranged V_HDJ_H allele in B-CLL cases.

V_HDJ_H rearranged allele was amplified using a forward primer annealing to the variable region in combination with a reverse primer specific for the 3' switch mu region.

Case 12, that did not present rearrangements in the $S\mu$ region, was used as control.

Chapter 6

DISCUSSION

The present thesis examines closely the immunoglobulin heavy chain (IgH) switch regions and variable regions in two hematological B-cell tumors, Mucosa-Associated Lymphoid Tissue (MALT) lymphoma and B-cell Chronic Lymphocytic Leukemia (B-CLL).

6.1 MALT lymphoma IgH switch regions

MALT lymphoma cells are thought to derive from marginal zone B-cells that are typically $CD5^-$, $CD10^-$ and IgM^+IgD^- (Harris et al., 1994; Wotherspoon, 1998). In order to define the differentiation of MALT lymphoma cells, numerous investigators sequenced the Ig variable heavy chain (V_H) genes and found frequent somatic hypermutations that could be ongoing (Hallas et al., 1998; Qin et al., 1997). Somatic hypermutations, together with isotype switch recombination, contribute to the process of B-cell maturation. Intrigued by the ability of MALT lymphoma cells to undergo somatic hypermutation of the variable genes and maintain in the majority of cases an "M" isotype, we investigated the ability of seven gastric MALT lymphoma to undergo DNA rearrangements within or near IgH switch regions.

Using a specific set of probes for the IgH switch regions we found by Southern blot that three out of five low-grade cases and two out of two high-grade cases showed

rearrangements within IgH switch regions, that appeared aberrant in four of the five cases. The cloning of four rearranged fragments from two low-grade and one high-grade confirmed the aberrant nature of the rearranged fragments.

In the low-grade **case n° 2** the two aberrant fragments detected by the 5'Sμ probe were cloned and characterized. The 4.7 kb aberrant fragment was generated by a deletion from the switch μ region (Sμ) to the first constant μ exon (Cμ 1) and a second deletion from the second constant μ exon (Cμ 2) to the gamma 3 region (γ 3) (Fig. 3.3). Since the first breakpoint clearly occurred in the Sμ region, it was probably generated by an error during the process of isotype switch recombination. Indeed, the molecular mechanisms of isotype switch recombination, V(D)J-recombination and somatic hypermutation are now becoming clearer and it is known that these events involve DNA double strand breaks. On the contrary, the mechanisms occurring during other type of recombinations are not yet known; for example, what happens to the partner chromosome during translocations involving the IgH locus (Kuppers and Dalla-Favera, 2001). Therefore the second breakpoint, which does not involve any switch region, might have been generated by an aberrant recombination other than a switch recombination. This aberrant recombination would have been facilitated by increased accessibility due to the first attempt to undergo a DNA switch to γ3 isotype.

The cloning of the 3.0 kb aberrant fragment detected by the 5'Sμ probe allowed the identification of the human switch alpha two (Sα2) region, which had never been sequenced before. To our knowledge only a short part of the human Sα2 sequence, localized upstream of the region identified in this project, was deposited in the GenBank (HUMIGHATC). It is not excluded that the Sα2 sequence obtained from this low-grade MALT lymphoma might contain somatic mutations due to the tumor

origin of the starting DNA. Moreover, although this case is IgM-producing, rearrangements involving the Sα regions are not surprising for a B-cell lymphoma associated with mucosal tissue which, is normally enriched with IgA antibodies. The 2.8 kb allele is possibly the non functional allele, while the 4.5 kb allele is the productive one. Indeed during the attempt to undergo an isotype switch recombination two DNA deletions occurred on this allele which, do not involve the region for the transcription of the V(D)J-Cμ message. Therefore, the 4.5 kb allele would still be able to produce a functional IgM antibody.

The cloning and sequencing of the aberrant fragments in **case n° 3** (low-grade) revealed that chromosome 14 undergoes a DNA recombination resulting in a partial deletion of the Sμ region. It has been suggested that a similar internal deletion or rearrangement of the Sμ region can occur in IgM-producing B cells to stabilize the isotype of the expressed immunoglobulin (Zhang et al., 1995).

In the 3'Sμ aberrant fragment of **case n° 7** (high-grade), a double recombination event occurred on chromosome 14q32: a deletion of the IgH intronic enhancer (Eμ) determined by a cut from J_H6 to Sμ and a 336bp insertion into the Sμ region of a gene (KIAA0307) normally located at 15q24 (Fig. 3.7a). As far as the insertion into the Sμ region is concerned it is possible to hypothesize that, it occurred during an attempt to undergo an isotype switch recombination. A dysregulation of the inserted KIAA0307 gene by the IgH 3' LCR is not likey since the insertion involves an intronic sequence of the gene. Much more interesting for the pathogenesis of the tumor is the deletion from chromosome 14 of the IgH intronic enhancer (Eμ) which, together with Eα1 and Eα2 located downstream of the alpha genes, has an essential

regulatory function for the expression of immunoglobulins. During chromosome translocations involving the IgH locus, the 3' enhancers may remain on the derivative chromosome 14 or may be dissociated but in both cases they have the potential to dysregulate juxtaposed oncogenes. It is possible to assume that in case 7 the IgH Eμ moved somewhere else in the genome, where, as previously reported for mouse plasmacytoma (Corcoran et al., 1985), it dysregulated an oncogene. Alternatively the Eμ might have been simply deleted from the cell. Future experiments will be planned to localize precisely the deleted intronic Eμ in this case.

The cloning of the four aberrant fragments and the discovery that they derive from alterations of the switch regions implied that switch aberrant fragments were generated by mistakes of the cell during an attempt to undergo a physiological isotype switch recombination. Many B-cell malignancies produce errors during the developmentally regulated processes of V(D)J-recombination, isotype switch recombination and somatic hypermutation (Kirsch, 1993; Korsmeyer, 1992). By analyzing IgH locus rearrangements within the V(D)J or switch regions and by considering the status of V(D)J hypermutation, it is possible to estimate the stage of B-cell development at which malignant transformation occurred (Matolcsy et al., 1997). For example, Mantle Cell Lymphoma, a tumor of virgin $sIgM^+$ B-cells, does not show V(D)J hypermutation or switch recombination and is characterized by a recurrent t(11;14)(q13;q32) occurring within J_H regions adjacent to heptamer-nonamer recognition sequences (Meeker et al., 1989; de Boer et al., 1993). Follicular Lymphoma, deriving from surface IgM^+ B-cells, shows ongoing V(D)J hypermutations and is characterized by a typical t(14;18)(q32;q21) occurring within J_H exons (Rack et al., 1998; Wu et al., 1994; Tsujimoto et al., 1985). In Burkitt's

Lymphoma, there is evidence of ongoing V(D)J somatic mutations. Furthermore, these tumors are characterized by a t(8;14)(q24;q32) with breakpoints within J_H exons (endemic form) or within Sμ regions (sporadic form) (Neri et al., 1988; Chapman et al., 1996; Magrath, 1990). Finally, Multiple Myeloma is a tumor of mature plasma cells which clearly undergo V(D)J somatic mutations and isotype switch recombination. Typical transclocations are promiscuous and may involve all switch regions (Bergsagel et al., 1996). MALT lymphoma cells show V(D)J hypermutations that can be ongoing (Hallas et al., 1998; Qin et al., 1997) and, as demonstrated in this work, can undergo IgH switch recombination. Therefore, it is possible to suggest that IgM^+ MALT lymphoma cells which do not show evidence of a switch recombination possibly originate from a less differentiated B-cell.

On the contrary it is not possible to determine if these aberrant recombinations occurred before or after the onset of malignancy. In the first case they would be involved in the development of the tumor, in the second case in the progression. In Multiple Myeloma it was possible to estimate the timing of occurrence of translocations, dividing them into primary translocations and secondary translocations (Bergsagel and kuehl, 2001) Through future studies of IgH rearrangements it will be possible to understand if some rearrangements are more frequent in low-grade than in high-grade MALT lymphomas.

Experiments on IgH switch regions demonstrate that the frequency of IgH switch rearrangements, other than normal class switching, has been grossly underestimated by other studies. The Southern blotting assay allowed us to estimate that aberrant switch recombination is a frequent event in MALT lymphomas.

Despite the very limited number of patients, the presence of aberrant switch recombination in low-grade lymphomas appeared to be associated with clinical

outcome. Cases without evidence of aberrant switch recombination had a shorter median disease free time after gastrectomy (6 months) compared to those with illegitimate switch recombinations (up to 56 months).

The cloning of a t(1;14)(p22;q32) translocation breakpoint from one case of MALT lymphoma allowed the identification of a new gene (Bcl10), which is also altered in other tumor types (Willis et al., 1999). Bcl10 is a novel apoptotic signalling gene that encodes an amino-terminal caspase recruitment domain and is overexpressed and truncated in MALT tumors carrying the t(1;14)(p22;q32) (Zhang et al., 1999). Mutant Bcl10 overexpression would provide anti-apoptotic and proliferative signals and confer a survival advantage to MALT B-cells.

During work described in this thesis, Bcl10 was never detected probably because the t(1;14) is a rare event in MALT lymphoma. However, the precedent set by the investigation of the t(1;14) translocation, suggested that the identification of aberrant switch recombination fragments in MALT lymphoma could give insights into the potential pathogenic role of new dysregulated genes.

6.2 MALT lymphoma variable genes

As shown by previous studies on switch regions, aberrant isotype switch events occur in an important proportion of gastric MALT lymphomas. Since isotype switch recombination and somatic hypermutation of immunoglobulin variable genes are markers of B cell maturation, gastric MALT lymphomas with or without aberrant rearrangements in the switch regions were examined for differences in maturation. First, as determined by Southern blot analysis of six gastric MALT lymphoma cases, aberrant isotype switch events which were found in three cases (Fig. 4.1). Then the

six cases were analyzed for the presence and type (common or non-common) of somatic hypermutations. Although it was supposed that somatic hypermutations may occur outside germinal centers it is well established that they occur usually in germinal centers (Davila et al., 2001). Moreover the detection of intraclonal diversification allows discrimination between germinal centers B cells, which show non-common mutations, and post germinal center B cells which do not (Kuppers et al., 1993).

Somatic common mutations were present in the immunoglobulin variable heavy chain genes of all six cases and homology with the closest germline ranged from 89.5 to 98.8%. A biased usage of the V_H 4 family and over-usage of D4-17 and of J_H4 segments was observed. An over-usage of a specific V_H family (V_H1-69), has been reported only in salivary gland MALT lymphomas (Miklos et al., 2000) while a predominant usage of J_H4 segment in gastric MALT lymphomas has been previously observed (Bertoni et al., 1997; Qin et al., 1995; Qin et al., 1997; Thiede et al., 1998). The clonal V_H genes of all six low-grade MALT lymphomas were potentially functional because no stop codons were present and the CDR3 sequences were all in-frame. Further analysis was performed to assess whether the mutation rate was due to antigen-driven selection. In the absence of negative or positive selection on a gene product, somatic hypermutations yielding amino acid replacement (R mutations) and not yielding amino acid replacement (S mutations) are randomly distributed throughout the coding sequence. A lower than expected frequency of R mutations indicates the pressure to mantain the protein sequence due to essential functions. By contrast if the number of R mutations exceeds that expected by chance alone, a positive selection process can be inferred. In four cases, the R/S ratios in the CDRs and FRs were characteristic of positively-selected or antigen-selected antibodies

which are under positive pressure to provide the "best fit" for antigen (Chang and Casali, 1994). On the other hand, two cases displayed R/S ratios that suggest selection by an autoantigen (Bahler et al., 1992; Friedmann, 1991; Friedman et al., 1991), and the CDR3 translated sequences in these two cases showed homology to autoantibodies. Surprisingly, two cases showed similar CDR3 sequences suggesting the presence of a common selecting antigen, as previously described in gastric MALT lymphomas by Bertoni et al. (Bertoni et al., 1997). The high R/S ratios in CDRs and low R/S ratios in FRs or the homology with autoantibodies coupled with the presence of intraclonal diversification, confirm the positive selective pressure of an antigen that plays a fundamental role in the pathogenesis of this disease, especially during the low-grade stage (Greiner et al., 1994; Hussell et al., 1993).

Isotype switch and V_H genes analysis in the six cases revealed several characteristics that suggest the existence of two distinct subsets of gastric low-grade MALT lymphoma.

First, high levels of intraclonal diversification were found in two of three cases without isotype switch events.

Secondly, lower levels of common mutations (2.4% on average) were found in cases without aberrant isotype switch events than in cases with such switch events (7.8% on average). Thus, one subset of gastric low-grade MALT lymphomas is characterized by V_H intraclonal diversification, few common mutations, and no aberrant isotype switch, whereas the other subset is characterized by the absence of V_H intraclonal diversification, many common mutations, and aberrant isotype switch. It is possible that the first subset originated from a germinal center environment and the second subset from a post-germinal center. Alternatively the initial transforming event might have occurred within the germinal center and additional maturation or

transforming events blocked the mutational mechanism. Further studies are needed to determine whether these two subsets have prognostic relevance and enable better evaluation of therapeutic modalities.

6.3 B-CLL analysis of IgH switch regions

B-cell Chronic Lymphocytic Leukemia (B-CLL) cells express mostly unswitched immunoglobulins (IgM or IgM/IgD), although non-productive rearrangements in the heavy chain switch mu (Sμ) have been detected in a proportion of cases (Crossen and Morrison, 1998; Laffan and Luzzatto, 1992; Hakim et al., 1993). Thirtyeight cases of B-CLL were investigated for the presence of non-productive rearrangements in the Sμ region and, for the first time, the molecular nature of all these rearrangements was defined. Southern blot analysis revealed Sμ region rearrangements in 13 cases (34%) and polymerase chain reactions (PCRs) indicated that these rearrangements consisted of internal deletions in the Sμ region. Long-distance PCRs localized the Sμ deletions in the $V_H(D)J_H$-rearranged allele in eight cases and in the unrearranged allele in only one case. Therefore most of these Sμ region rearrangements, previously observed in B-CLL by other investigators, are genomic deletions of the Sμ region occurring in most cases in the $V_H(D)J_H$-rearranged allele. These deletions are of variable length, ranging from 0.5 kb to about 4 kb and may involve the region upstream or downstream the Sμ region but never the constant μ exons. The random distribution of these deletions might be related to the absence of a consensus-cutting site inside the Sμ region (Dunnick et al., 1993; Kinoshita et al., 1998). In two cases (cases n° 3 and 31), the Sμ deletion occurred on both alleles which is not surprising since there is no evidence suggesting that the switch mechanism can distinguish between the

productive and non-productive allele in a B cell (Sakai et al., 1999). However, Sμ deletions are in most cases found within the $V_H(D)J_H$-rearranged allele, thus strongly supporting the hypothesis that B-CLL cells have undergone this partial deletion of the Sμ region in order to stabilize the isotype of the expressed immunogloblin (Mukherjee et al., 1993; Zhang et al., 1995). Indeed, although it was demonstrated that the Ig heavy chain intronic enhancer is necessary and sufficient to promote class switch recombination (Sakai et al., 1999), a deletion of the Sμ tandem repeats in the mouse immunoglobulin heavy chain locus reduces the efficiency of class switching. The hypothesis of isotype stabilization is not incompatible with the presence of isotype-switched transcripts, previously described in IgM^+ B-CLL cells (Malisan et al., 1996). As mentioned above, even a cell with Sμ deletion can undergo isotype switch recombination, although with lower efficiency.

Sμ internal deletions might also represent "failed switching attempts", as suggested by Q. Pan *et al.,* who frequently detected internal deletions in the Sμ, Sγ4 and Sγ3 regions. (Pan and Hammarstrom, 1999). Possibly, "failed switching attempts" may themselves contribute to isotype stabilization. This mechanism can explain why only a small proportion of B-CLL cells undergo isotype switch and might be highly relevant to the process of isotype switching in this leukemia. It is unknown if Sμ deletions occur in a B cell before or after malignant transformation; however, the demonstration that these rearrangements are Sμ internal deletions and not chromosome translocations rules out their involvment in the onset of malignancy.

6.4 B-CLL variable genes

For a long time, it was assumed that the processes of isotype switch recombination

and somatic hypermutations of the variable genes were unrelated phenomena (van Es et al., 1992; Wysocki et al., 1992; Gilmore et al., 1987) and indeed it is possible to have IgM^+ cells with V_H mutations (Schettino et al., 1998) and IgG^+/A^+ cells without V_H mutations (Matolcsy et al., 1997). It appears, however, that these two developmentally regulated processes occur in temporal correlation (Wabl et al., 1999) and new links between them are becoming clearer day by day (Kinoshita and Honjo, 2001).

Intrigued by this relation, we investigated if Sμ deletions are related to V_H somatic mutations in B-CLL. Sequencing of the V_H regions of all 38 cases indicated the presence of somatic hypermutations in the V_H genes of 17 cases (44,7%). In one case, two productive V_H genes were expressed, which is not infrequent, since about 5% of B-CLL cells lack immunoglobulin heavy chain allelic exclusion (Rassenti and Kipps, 1997). Of the two productive V_H genes, one was mutated and the other was not. Of interest, cases with V_H mutations showed overusage of the V_H3 family while cases without V_H mutations showed overusage of the V_H1 family. Overusage of the V_H1 family by B-CLL leukemic cells has been extensively reported (Schroeder and Dighiero, 1994; Maloum et al., 2000; Fais et al., 1998; Kipps et al., 1989) while only recently overusage of the V_H3 family (in particular V_H3-21) has been associated with mutated V_H genes (Tobin et al., 2002). Biased VH gene usage might derive from chronic antigenic stimulation and play a role in the development of malignancy. In 8/17 cases with V_H mutations, it was indeed possible to postulate the effect of selection by an antigen since R/S ratios in the CDRs were higher than expected from random distribution and in FRs were lower (Maloum et al., 2000; Chang and Casali, 1994; Fais et al., 1998; Kipps et al., 1989; Schroeder and Dighiero, 1994). It is tempting to speculate on a possible role for antigens in the development of some B-

CLL; these antigens could bind specific epitopes encoded by the overused V_H gene and stimulate proliferation of the malignant clone. Future studies will be necessary to clarify the possible role of specific antigens in the pathogenesis of B-CLL.

No correlation between the presence of Sμ deletions and the presence of V_H gene mutations was observed, since 32% of cases with a deletion in the Sμ region showed V_H mutations, and 52% of cases without deletion in the Sμ showed V_H mutations. Therefore, Sμ internal deletions might occur independently of V_H mutations and considering the V_H status as a marker of B-cell maturation, Sμ deletions might occur independently of the B cell maturation. This is quite different from other genetic abnormalities in B-CLL, such 13q14 deletions which occur preferentially in "post-germinal center" memory B-cells and trisomy 12 in "pre-germinal center" naïve B-cells. An additional hypothesis is that all cases of B-CLL do not derive from B cells with different maturation but from a cell with the unique features of a "memory B cell". Indeed recent gene expression profile analyses showed that B-CLL display a common and characteristic gene expression profile that is largely independent of their IgV genotype and that resembles features of memory B cells (Klein et al., 2001; Rosenwald et al., 2001).

Moreover, while a correlation of V_H mutations with disease progression was detected, confirming recent data on the possible use of V_H status as a prognostic factor (Hamblin et al., 1999), Sμ rearrangements alone did not appear to be indicative of clinical course. Together these data indicate that in B-CLL Sμ internal deletions occur independently of V_H somatic mutations and have apparently no consequence for disease outcome.

The four following publications were derived from work presented in this thesis:

1 E.Nardini, S.Rizzi, S.Menard, A.Balsari. "Molecular phenotype distinguishes two subsets of gastric low-grade Mucosa-Associated Lymphoid Tissue (MALT) lymphomas" *Laboratory Investigation* 2002 May; 82 (5): 535-42.

2 E.Nardini, S.Rizzi, D.Capello, G.Gaidano, U.Vitolo, S.Menard, A.Balsari. "Most immunoglobulin heavy chain switch mu rearrangements that occur in unswitched B-cell Chronic Lymphocytic Leukemia are internal deletions" *Febs Lett.* 2002 May 8; 518 (1-3): 119-123.

3 Nardini E, Rizzi S, Menard S, Balsari A. "Identification of the human switch alpha 2 region from a low-grade malt lymphoma." *Mamm Genome*. 2000 Dec;11(12):1145-6.

4 Nardini E, Aiello A, Giardini R, Colnaghi MI, Menard S, Balsari A. "Detection of aberrant isotype switch recombination in low-grade and high-grade gastric MALT lymphomas." *Blood.* 2000 Feb 1;95(3):1032-8.

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